REMARKS

Pending Claims

Before entry of the foregoing amendments, Claims 1-10 are pending in the above-captioned application. Claims 1-10 are directed to a method of inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell.

The Office Action, Applicant's Response and Amendment

The Examiner acknowledged Applicant's election, in Paper No. 11, of designated claim Group I (Claims 1-10), without traverse, in response to a restriction requirement. The Examiner acknowledged the entry of Applicant's amendment cancelling Claim 11-23, without prejudice, and with a reservation of all rights under 35 U.S.C. § 121.

The Examiner noted that if Applicant desires priority under 35 U.S.C. § 120 or § 119(e) based upon a previously filed copending application, specific reference to the earlier filed application must be made in the instant application and that this reference should appear as the *first sentence* of the specification following the title, preferably a separate paragraph, not as in the application as filed in which the reference regarding the claimed priority is in the second paragraph. Appropriate correction was required. Accordingly, Applicant has inserted the reference to priority applications at page 1, line 4 *et seq.* and has deleted the reference at page 1, lines 8-15. Further, the continuing data have been updated with respect to U.S. Serial No. 08/894,251, filed on July 23, 1999, which issued as U.S. Patent No. 6,455,305, on September 24, 2002.

The Statement of U.S. Government rights has been inserted as the second paragraph, instead of as first paragraph.

The amendment to Claim 7 is merely refining for the sake of greater clarity.

The broadening amendment in Claim 8 is supported in the specification, e.g., at page 30, line 7 through through page 34, line 15, especially at page 31, line 8 through page 32 line 2; and page 32, line 30 through page 33, line 20.

New Claims 24-31 are added.

Support for Claim 24 is found in the specification as originally filed, e.g., in Claim 1; and at page 47, line 22 through page 48, line 4, especially page 47, line 30 through page 48, line 2.

Support for Claim 25 is found, e.g., in Claim 2, as originally filed.

Support for Claim 26 is found, e.g., in Claim 3, as originally filed.

Support for Claim 27 is found, e.g., in Claim 4, as originally filed.

Support for Claim 28 is found, e.g., in Claim 5, as originally filed.

Support for Claim 29 is found, e.g., in Claim 6, as originally filed.

Support for Claim 30 is found, e.g., in Claim 7, as originally filed.

Support for Claim 31 is found, e.g., in Claim 8, as originally filed; and in the specification at page 30, line 7 through through page 34, line 15, especially at page 31, line 8 through page 32 line 2; and page 32, line 30 through page 33, line 20.

Applicant believes that no new matter is introduced by any amendments made herein.

The Examiner acknowledged Applicant's claim for domestic priority under 35 U.S.C. § 119(e) and § 120, however, the Examiner stated that the provisional application 60/031,338 and applications 89/777,422, 89/730,469, 09/687,911, 09/569,956, 08/894,251 and PCT/US97/21463 fail to disclose the nucleotide sequence of SEQ ID No. 63 and the amino acid sequence of SEQ ID No. 64. Thus, the benefits of the provisional application 60/031,338 and applications 09/777,422, 09,730,469, 09/687,911, 09/569,956, 08/894,251 and PCD/US97/21463 were denied. The Examiner stated that the effective filing date of the present application is the actual filing date May 11, 2001.

The Examiner stated that the specification is "enabling for inducing neoplastic transformation by PTTG1 polypeptide and the proline-rich domain of PTTG1 is important for

PTTG-mediated neoplastic transformation, and overexpression of PTTG2 inhibits transactivation activity of PTTG1 by nearly half in vitro."

However, no claims were allowed.

The Examiner asserted the following grounds of rejection.

A. Rejections under 35 U.S.C. § 101

Claim 9 was rejected under, under 35 U.S.C. § 101 [and 35 U.S.C. § 112, first paragraph], for the following reason stated by the Examiner:

Claim 9 is rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific asserted utility or a well established utility.

The specification fails to provide an asserted use that meets the requirement of 35 U.S.C. 101 for inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell in vitro. There is no evidence of record for a well-established utility for inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell in vitro. There is no specific utility or a well-established utility for the mammalian cells whose neoplastic cellular proliferation and/or transformation has been inhibited. The only readily apparent use for the method is to study the effects of the method. The use of an invention as an object of further research or study does not meet the requirement of 35 U.S.C. 101.

Claim 9 is also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

The rejection is mooted by the cancellation of Claim 9, the subject matter of which the Applicant reserves the right to claim in a continuation application, and the amendment to the preamble of Claim 1, limiting the claimed method of inhibiting neoplastic cellular proliferation and/or transformation, or both, of a mammalian cell, *in vivo* . . ." In view of this amendment to Claim 1, Applicant has canceled Claim 10 as merely being redundant.

Consequently, the Examiner is respectfully requested to withdraw the rejection on this ground.

B. Rejections under 35 U.S.C. § 112, second paragraph

Claims 1-10 were rejected, under 35 U.S.C. § 112, second paragraph. The Examiner stated the following reason:

The term "and/or" in claims 1-3 is vague and renders the claims indefinite. It is unclear what is intended to be claimed. Changing the term "and/or" to "...or...or both" would be remedial. Claims 4-10 depend on claim 1 but fail to clarify the indefiniteness.

In response, Applicant has made appropriate, merely formal refining amendments, to Claims 1, 2, and 3, concerning "and/or", which amendments do not narrow the scope of the claims. Consequently, the Examiner is respectfully requested to withdraw the rejection of Claims 1-8 (Claims 9-10 being canceled) on this ground.

C. Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-8 and 10 were rejected under 25 U.S.C. § 112, first paragraph, for the following reasons:

... the specification, while being enabling for inducing neoplastic transformation by PTTG1 polypeptide and the proline-rich domain of PTTG1 is important for PTTG-mediated neoplastic transformation, and overexpression of PTTG2 inhibits transactivation activity of PTTG1 by nearly half in vitro, does not reasonably provide enablement for a method of inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell by delivering a composition comprising any expression vector expressing a mammalian PTTG2 peptide to a mammalian cell via any administration route in vivo, where said PTTG2 peptide consists essentially of amino acid residues 1-191 of SEQ ID No. 64 or a functional fragment thereof comprising at least 1-180 of SEQ ID No. 64, or a mammalian PTTG2 peptide having at least 95% identity to the PTTG peptide set forth above. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Claims 1-8 and 10 are directed to a method of inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell, such as a malignant cell, by delivering a composition comprising an expression vector expressing a mammalian PTTG2 peptide to a mammalian cell in vitro or in vivo, wherein said PTTG2 peptide consists essentially of amino acid residues 1-191 of SEQ ID No. 64 or a functional fragment thereof comprising at least 1-180 of SEQ ID No. 64, or a mammalian PTTG2 peptide having at least 95% identity to the PTTG peptide set forth above, and said expression vector is complexed with a cellular uptake-enhancing agent.

The specification discloses that stable expression of human PTTG1 polypeptide in transfected NIH3T3 cells induces tumor formation in vitro and in vivo, and the proline-rich domain of PTTG is important for PTTG-mediated neoplastic transformation. Human PTTG2 polypeptide is 90% identical to human PTTG1 polypeptide for 179 amino acid residues and its carboxyl terminal is non-homologous for an additional 12 amino acid residues, and removal of the carboxy terminal segment increases transactivation activity by 26 folds. The specification also discloses that overexpression of PTTG2 inhibits transactivation activity of PTTG1 by nearly half in vitro (specification, page 116). The claims encompass inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell by delivering a composition comprising any expression vector expressing the claimed mammalian PTTG2 peptide to a mammalian cell via any administration route in vivo.

The specification fails to provide adequate guidance and evidence for a method of inhibiting neoplastic cellular proliferation and/or transformation of a mammalian cell by delivering a composition comprising any expression vector expressing the claimed mammalian PTTG2 peptide to a mammalian cell via any administration route in vivo such that therapeutic effect can be obtained in a subject.

The claims read on gene therapy in vivo. The state of the art for gene therapy was unpredictable at the time of the invention. While progress has been made in recent years for gene transfer in vivo, vector targeting to desired tissues in vivo continues to be unpredictable and inefficient as supported by numerous teachings available in the art. For example, Deonarain (1998, Expert Opin. Ther. Pat., Vol. 8, pages 53-69) indicates that one of the biggest problems hampering successful gene therapy is the "ability to target a gene to a significant population of cells and express it at

express it at adequate levels for a long enough period of time" (page 53, first paragraph). Deonarain reviews new techniques under experimentation in the art which show promise but states that such techniques are even less efficient than viral gene delivery (see page 65, first paragraph under Conclusion section). Verma (Sept. 1997, Nature, Vol. 389, pages 239-242) reviews vectors known in the art for use in gene therapy and discusses problems associated with each type of vector. The teachings of Verma indicate a resolution to vector targeting has not been achieved in the art (see entire article). Verma also teaches appropriate regulatory elements may improve expression, but it is unpredictable what tissues such regulatory elements target (page 240, sentence bridging columns 2 and 3). Verma states that "The Achilles heel of gene therapy is gene delivery, and this is the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression....The use of viruses (viral vectors) is powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells, However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses." (e.g. p. 239, column 3).

Further, Eck et al., 1996 (Goodman & Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, p. 77-101) states that the fate of the DNA vector itself (volume of distribution, rate of clearance into the tissues, etc.), the in vivo consequences of altered gene expression and protein function, the fraction of vector taken up by the target cell population, the trafficking of the genetic material within cellular organelles, and the rate of degradation of the DNA, the level of mRNA produced, the stability of the mRNA produced, the amount and stability of the protein produced, and the protein's compartmentalization within the cell, or its secretory fate, once produced are all important factors for a successful gene therapy (e.g. bridging pages 81-82). In addition, Gorecki, 2001 (Expert Opin. Emerging Drugs, 6(2): 187-198) reports that "the choice of vectors and delivery routes depends on the nature of the target cells and the required levels and stability of expression" for gene therapy, and obstacles to gene therapy in vivo include "the development of effective clinical products" and "the low levels and stability of expression and immune responses to vectors and/or gene products" (e.g. abstract). In view of the lack of adequate guidance and evidence and the unpredictability in gene transfer as discussed above, one skilled in the art at the time of the invention would not know how to use various vectors comprising the polynucleotide encoding the claimed mammalian PTTG2 peptide to inhibit neoplastic cellular proliferation and/or transformation of a mammalian cell via any administration route in vivo so as to provide therapeutic effect in a subject.

For the reasons discussed above, it would have required undue experimentation for one skilled in the art at the time of the invention to practice over the full scope of the invention claimed. This is particularly true given the nature of the invention, the state of the prior art, the breadth of the claims, the amount of experimentation necessary, the working examples provided and scarcity of guidance in the specification, and the unpredictable nature of the art.

Applicant agrees with the Examiner that gene therapy is a relatively new art and is still in the process of being developed over the last decade. Nevertheless, "gene therapy has also achieved success in four early stage clinical trials -- each one surprisingly using a different delivery system. We have witnessed the treatment of severe combined immunodeficiency with a retroviral vector, hemophilia with an adeno-associated viral vector, cardiovascular disease with naked plasmid DNA and cancer therapy using an oncolytic adenovirus." (Anderson, W.F., Gene therapy scores against cancer, Nat. Med. 6(8):862-63 [August 2000], page 862, last paragraph [citations omitted]; appended as **Exhibit A**). As of March 2000, about 350 gene therapy clinical trials had been conducted or were underway. (**Exhibit A**: Anderson, page 862, column 3). In order to reach the stage of human clinical trial status, however, pre-clinical trials have had to be at least partially successful, and this has required a large body of good reliable data on animal models and vectors for mammalian gene therapy. At the time the

present specification was filed, there was already a successful model of in utero gene therapy in a large mammal, which produced xenogeneic expression over at least 5 years without pathology. (E.g., Porada *et al.*, *In utero gene thereapy: transfer and long-term expression of the bacterial neo^r gene in sheep after direct injection of retroviral vectors into preimmune fetuses*, Human Gene Therapy 9:1571-85 [July 20, 1998]; appended as **Exhibit B**). Even at the time the present application was filed, gene therapy in humans had had some notable successes, including long-term in vivo therapeutic expression of exogenous genes and clinical improvement in a human suffering from adenosine deaminase deficiency. (Mullen, C.A. *et al.*, *Molecular analysis of T lymphocyte-directed gene therapy for adenosine deaminase deficiency: long-term expression in vivo of genes introduced with a retroviral vector*, Human Gene Therapy 7:1123-1129 [June 1996]; copy appended as **Exhibit C**).

As noted by the Examiner, the Gorecki reference, discusses certain obstacles in gene therapy in vivo that must be considered, but Gorecki fails to teach that these obstacles prevent successful gene therapy. For example, the Gorecki reference describes "exciting developments" in gene therapy in recent years (e.g., pp. 194-95) and the hundreds of *clinical* trials being conducted with thousands of patients (e.g., pp. 193-94, bridging paragraph), which, Applicant submits, confirms indeed a relatively high level of predictability and enablement known in the art, sufficient for patent purposes.

Applicant's claimed method is not limited to employing any particular expression vector system, and can be practiced using a variety of available expression vector systems that can be constructed to include ". . . a polynucleotide . . . comprising a first DNA segment encoding a mammalian PTTG2 peptide," as recited in Claim 1. Contrary to the Examiner's assertion, delivery of the expression vector ". . . such that the PTTG2 peptide is expressed in the cell," in accordance with the claimed method would indeed have been enabled by the disclosure of the specification and the general knowledge in the art, and success would not have been made unpredictable by the problems described by the Examiner, such as targeting, lack of expression, and immune clearance.

At the time the above-captioned application was filed, the skilled artisan would have known of means for delivering an expression vector with the ability, if desired, of particularly targeting neoplastic cells and tissues, with respect to the claimed method of inhibiting neoplastic cellular proliferation or transformation, or both, of a mammalian cell (e.g., Claim 1). For example, injectable pseudotyped retroviral vectors were known that selectively target collagen in the extracellular matrix, which is exposed in neoplastic tissue, since tumor invasion, as well as angiogenesis and stroma formation, evoke a remodeling of extracellular matrix components and exposure of collagen. (**Exhibit D**: Hall, F.L. *et al.*, *Molecular Engineering of matrix-targeted retroviral vectors incorporating a surveillance function inherent in von Willebrand factor*, Human Gene Therapy 11:983-993 [May 1, 2000], see, e.g., Abstract). Selective transduction of tumor cells *in vivo* within metastatic tumor foci is observed after delivering such a vector by intravascular infusion. (See, e.g., **Exhibit D**: Hall *et al.*, at page 989, first column, first paragraph; at page 990, first column through page 992, first column, end of bridging paragraph). The skilled artisan would also have been aware of means for constructing expression vectors with the capacity for sustained tumor-specific expression, if desired, for example, by incorporating hypoxia-responsive elements (HREs) along with a constitutive CMV or other promoter, which could selectively express an exogenous gene in neoplastic cells in tumor tissues, which tend to be hypoxic compared to normal tissues. (E.g., **Exhibit E**: Shibata, T. *et al.*, *Development of a hypoxia-responsive vector for tumor-specific gene therapy*, Gene Therapy 7:493-98 [2000], see, e.g., Abstract). The above are merely non-exhaustive examples of expression vector constructs with which the skilled artisan would have been able to overcome the problems that the Examiner has raised.

Finally, with respect to the Examiner's assertion that the human immune system will prevent the effective delivery of DNA even by viral vectors, Applicant submits a number of pre-filing date references, substantially more recent than the cited Verma and Eck *et al.* references, that show that effective systemic delivery of exogenous DNA is reasonably predictable in immunocompetent subjects. For example, Rainov *et al.* (Cancer Gene Ther. 5(3):158-62 [1998]; abstract appended as **Exhibit F**) describes *intraarterial* (intracarotid) delivery of HSV-1 mutant hrR3 vector + gancylovir to intracerebral 9L tumors (gliosarcoma)

in *immunocompetent* Fischer (F344) rats. Their procedure resulted in eradication of tumors in 80% of test animals compared to 100% mortality in the control group. The Rainov *et al.* (1998) reference also shows that it was known in the art that increasing the dose of a viral vector (e.g., in **Exhibit F**: Rainov *et al.* [1998]: 10^{10} pfu versus 10^{9} or 10^{8} pfu) could improve results. This is an indication that even were host immune response a significant factor, it could be predicatbly overcome with increased vector dose to ensure adequate gene delivery to target cells via a systemic administration route.

Another example, Rainov *et al.*, Human Gene Ther. 10(2):311-8 (Jan 1999; appended as **Exhibit G**) showed a similar result using a different viral vector (replication-defective adenovirus vector), which reference tends to confirm the predictability of successful genetic transfer using systemically delivered recombinant viral vectors. Again, this reference demonstrated successful *intrarterial* delivery of viral vector to target cells (9L brain tumors) in *immunocompetent* Fischer (F344) rat hosts.

Still another reference, Herrlinger *et al.*, Gene Ther. 5(6):809-19 (1998; abstract appended as **Exhibit H**), also describes treatment of intracranial tumors in *immunocompetent* Fischer rats with the HSV-1 mutant hrR3. Although this reference disclosed direct intratumoral injection of vector and not systemic delivery, the reference demonstrates that even preimmunization of the mammalian subject with HSV-1 fails to abolish gene transfer.

By way of further support of Applicants' assertion that immune clearance of vector does not prevent enablement of the claimed method, Applicants submit herewith as **Exhibit I**: Ikeda *et al.*, Nature Medicine 5(8):881-87 (August 1999), which confirms the predictability of vector delivery by routes other than in situ delivery. Ikeda *et al.* describes immune response against hrR3 HSV-1 mutant vector in *immunocompetent* Fischer rats. The rats formed neutralizing antibodies 4 days (Figure 1) after intraarterial injection of hrR3 vector, which response was partially abrogated by simultaneous treatment with the immunosuppressant cyclophosphamide. Consequently, Ikeda *et al.* stated that "intraarterial administration should result in initial infection of some cells in the tumor, with initial generation of progeny viruses (12-18 hours) and subsequent rounds of infection (24-48 hours)." (At page 881, col. 2, first full paragraph). Moreover, Ikeda *et al.* states that

"neutralizing antibody responses, detected at 4 days, are not likely to affect these early processes." Even a significant percentage of control rats that were not treated with immunosuppressant cyclophosphamide showed an increase in survival when injected with the hrR3 HSV-1-derived vector, compared to mock vehicle control (e.g., Fig. 6b).

In view of the above, Applicants' claimed method is fully enabled for systemic delivery routes as well as in situ delivery. Even if a strong immune response occurs in a mammalian host, a vector, in accordance with the claimed method, can predictably reach target cells before immune clearance (see, e.g., **Exhibit I**: Ikeda *et al.* [1999]), and, regardless, the skilled artisan would know that the dose of vector can be manipulated to overcome an immune response (See, e.g., Rainov *et al.* [1998]).

Therefore, it is reasonably predictable, based on disclosures of Applicant's specification and the general knowledge in the art that, in accordance with the claimed methods, the HSV-1 derived vector can be delivered by routes other than by direct intratumoral injection without immune clearance that totally prevents gene delivery to target cells.

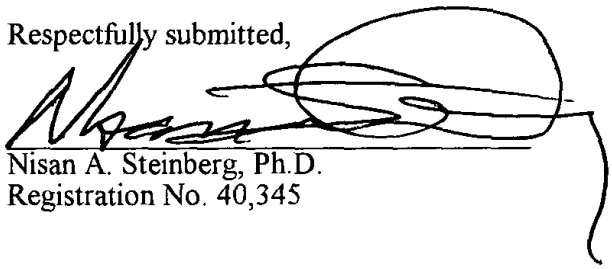
Applicant submits that while there may be room for improvements in therapeutic efficiency and while individual patient differences may result in variable responses as with any other therapy modality, gene therapy protocols and useful vectors (see, e.g., Verma *et al.*, page 240, third column through page 242, first column) are currently known for practicing gene therapy successfully and safely in at least a subset of patients, which is sufficient for patent purposes. The fact that some other patients may not respond well to a particular gene therapy protocol, does not make gene therapy a "highly unpredictable" art. Few, if any, therapy modalities are 100% effective in all cases.

In view of the above, Applicant respectfully requests that the rejection be withdrawn on this ground.

CONCLUSION

In view of the above amendments and remarks, it is submitted that this application is now ready for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney at (213) 896-6665.

Respectfully submitted,



Nisan A. Steinberg, Ph.D.
Registration No. 40,345

Sidley Austin Brown & Wood LLP
555 West Fifth Street, Suite 4000
Los Angeles, California 90013-1010
Telephone: (213) 896-6665
Facsimile: (213) 896-6600

Gene therapy scores against cancer

The first Phase II clinical trial to study the combination of a replicating viral agent with chemotherapy indicates that cancer gene therapy may finally be on the road to success. (879-885).

W. FRENCH ANDERSON

SQUAMOUS CELL CARCINOMA of the head and neck afflicts approximately 500,000 patients per year. The first-line treatment, chemotherapy, induces a response in only 30–40% of patients, and tumors frequently recur. Alternative cancer therapy approaches have been based on oncolytic viruses, which selectively attack tumor, but not normal, cells. One of these, ONYX-015, was initially tested in a Phase I clinical trial in April 1996. In this issue, Khuri *et al.* report that ONYX-015, when combined with chemotherapy, promotes tumor regression in patients with recurrent squamous cell cancer of the head and neck[1].

ONYX-015 is a modified adenovirus – a DNA virus that takes over the cell's protein synthesis machinery, replicates, then lyses the host cell to release its progeny. In wild-type adenovirus, the early regulatory protein E1B-55kDa binds to and inactivates the host cell's p53 protein[2] to promote its own replication. Without E1B-55kDa, adenovirus is incapable of replication[3]. However, researchers observed that an E1B-55kDa mutant adenovirus, ONYX-015, could replicate in and lyse p53-negative, but not p53-positive, human tumor cells[4]. As p53 is mutated in 45–70% of all cases of head and neck cancers, ONYX-015 was developed as a tumor cell-specific therapeutic agent.

Tumor-selective destruction has been observed in squamous cell carcinoma patients receiving ONYX-015; however, clinical benefit was observed in less than 15% of patients. Khuri *et al.* studied the effects of ONYX-015 in combination with standard chemotherapy[1]. 30 patients with recurrent squamous cell cancer of the head and neck were evaluated for their response to a combination of chemotherapy (cisplatin and 5-fluorouracil) and ONYX-015, which was injected directly into tumors[1].

The combined therapy was well tolerated and did not cause significant levels of toxicity. The treatment caused an objective response (at least a 50% reduction in tumor size) in 19 cases, with 8 complete responses[1]. Tumors as large as 10 cm in diameter regressed completely and none of the tumors that demonstrated an objective response had progressed after a mean follow-up of 5 months. The authors concluded that the tumor response rate, complete response rate and time to tumor progression rate after combined therapy were better than those observed after therapy with cisplatin and 5-fluorouracil alone. Furthermore, biopsy samples indicated that ONYX-015 replicates within tumor tissue but does not replicate in normal tissue.

The concept of using a gene therapy vector that selectively replicates in and kills tumor cells has been around for a number of years, but the regulatory concern of putting a replicating vector in patients prevented acceptance of the idea until recently. Now, a number of approaches based on this concept are being investigated. Replicating oncolytic viruses are advantageous because they would theoretically be able to reach widespread metastases. Furthermore, vectors such as ONYX-015 could probably be made even more effective if they incorporated suicide genes like herpes simplex thymidine kinase and/or cytosine deaminase.

The field of gene therapy has had a history of unexpected turns. ONYX-015 is another. The first irony in the field was the use of 'gene therapy' (actually gene transfer), not for a genetic disease as everyone expected, but for marking tumor-infiltrating lymphocytes in patients with malignant melanoma[5]. The first gene therapy clinical trial was for the genetic disease ADA deficiency in 1990 (ref. 6), and by March 1995, there were 32 trials for genetic diseases, including 16 for cystic fibrosis and 16 for a range of other monogenic diseases. Although it seemed that cystic fibrosis would be the first gene therapy success, hemophilia was the first genetic disease for which encouraging clinical data were reported[7].

Of the 277 gene therapy clinical trials reported by the National Institutes of Health Recombinant DNA Advisory Committee (NIH RAC) in May 1999, 70% were for treatment of cancer and only 5% were for treatment of cardiovascular diseases. Therefore, it seemed that cancer would be the first 'acquired' disease that would be successfully treated by gene therapy. However, researchers soon after announced that direct injection of the vascular endothelial growth factor gene induced new blood vessel formation in cardiovascular disease patients[8].

Cancer is still the disease most frequently targeted by gene therapy. According to the March 2000 NIH RAC database, of the 350 gene therapy clinical trials, 67% are for cancer. Of those, 31% use *in vitro* immunotherapies, 32% use *in vivo* immunotherapies, 15% are based on pro-drug suicide therapies, and only 2% are using a 'vector-directed cell lysis' approach. It is therefore ironic that the first anti-cancer clinical success has arisen from the last approach and that the vector does not even carry a 'therapeutic' gene.

So with all its twists and turns, gene therapy seems to be turning the corner after a very bad year. Not only has the field been criticized for too much hype and too few successes, but in September 1999, an 18-year old patient died as a direct result of a gene therapy intervention. But gene therapy has also achieved success in four early stage clinical trials – each one, surprisingly, using a different delivery system. We have witnessed the treatment of severe combined immunodeficiency with a retroviral vector[9], hemophilia with an adeno-associated viral vector[7], cardiovascular disease with naked plasmid DNA[8] and cancer therapy using an oncolytic adenovirus[1]. Although the increased oversight and new monitoring requirements will improve future gene therapy trials, what the field really needs are successes in Phase III trials.

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2. Barker, D.D. & Berk, A.J. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 156, 107–121

(1987).
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University of Southern California
Keck School of Medicine,
Los Angeles, California 90033
Email: sdiaz@genome2.hsc.usc.edu

Human cytomegalovirus - no longer just a DNA virus

Human cytomegalovirus (HCMV) is classified as a DNA herpesvirus. A recent study showed that the HCMV virion contains not only DNA, but also four species of mRNA, indicating that this virus is more complex than previously believed.

ENG-SHANG HUANG & ROBERT A. JOHNSON

HUMAN CYTOMEGALOVIRUS (HCMV) is the largest of the known herpesviruses, with a genome of over 235 kb and more than 200 potential open reading frames[1]. It is a widespread opportunistic pathogen that causes no obvious clinical manifestation in healthy individuals. On the other hand, it is a leading cause of virus-associated birth defects, including mental retardation and deafness. It causes severe and fatal diseases in immune-compromised individuals, such as organ transplant recipients and AIDS patients, and is also associated with atherosclerosis and coronary restenosis.

When HCMV infects a cell, the viral capsid is transported to the nucleus, where the viral DNA is released and transcribed by the host cell machinery (Fig. 1). It was commonly believed that all the newly synthesized viral proteins were encoded by the viral DNA genome[2]. However, in a recent issue of *Science*, Bresnahan and Shenk demonstrated that in addition to viral DNA, HCMV virus particles also carry mRNAs into the host cell[3]. Using gene array technology, they identified four virally encoded mRNAs that are present in highly purified HCMV virions. These mRNAs are delivered to the cell cytoplasm when the viral envelope fuses with the plasma membrane at the start of infection. The four mRNAs remain in the cytoplasm, where they are translated into proteins in the absence of gene products encoded by viral DNA. One of the mRNAs, UL21.5, encodes a protein that contains a leader sequence that localizes it to the Golgi network, but the function(s) of the mRNAs or their products is unknown.

HCMV genes are classified as immediate early, early or late, depending on the timing and conditions of their expression. Bresnahan and Shenk found that the virally encoded mRNAs are transcribed from one immediate early gene (UL106-109), two early genes (TRL/IRL2-5 and TRL/IRL7) and one late gene (UL21.5) (ref. 3). These mRNAs are transcribed from viral DNA and accumulate to highest levels at late times of infection, when the virions are being assembled. The association of these HCMV mRNAs with virion is specific, as the four viral transcripts were the only ones found to couple with the virion.

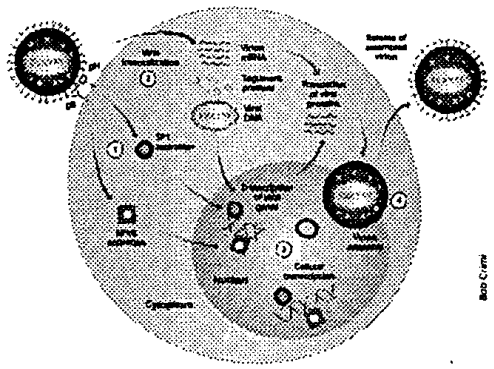

Fig. 1 HCMV lytic life cycle. *1*, Binding of HCMV glycoproteins gB and gH to cellular receptors activates cellular transcription factors, such as NF-κB and Sp1. *2*, The virus then enters the cell, releasing viral DNA, virion proteins and virion mRNA transcripts into the cytoplasm, where virion mRNAs are translated. Viral DNA and certain viral proteins are transported to the nucleus. *3*, In the nucleus, viral and cellular genes are expressed, with help from the activated transcription factors, and viral DNA is replicated. *4*, Viral DNA, viral and cellular proteins, and virion transcripts are packaged into the virion through an as yet undetermined mechanism. During egression of the virion from the cell, the virion envelope is constructed and an infectious viral particle is released.

The overall structure of the HCMV viral particle is similar to that of other herpesviruses. The outermost layer is the lipid bilayer envelope that contains cellular and virally encoded glycoproteins. At the core of the particle is the viral DNA that is surrounded by an icosahedral capsid. Between the capsid and the envelope is an amorphous layer referred to as the tegument. It is in this tegument region that the mRNAs described by Bresnahan and Shenk most likely reside[3].

The HCMV virion tegument also contains many proteins. These include DNA polymerase, protein kinase[4] and a cellular topoisomerase II enzyme (required for viral DNA replication)[5]. An Fc receptor for human immunoglobulin G is located within the tegument, along with the serine/threonine protein phosphatase PP1 and the host cell-derived phosphatase PP2A (ref. 6). The viral gene products UL82, UL69 and UL83 (refs. 7, 8) are also present in the tegument. These proteins are released into the cytoplasm after viral infection and are required for viral replication and virus-induced pathogenesis. For example, DNA polymerase and topoisomerase II are believed to enhance viral DNA replication. The UL82 gene product, pp71 (the HSV VP-16 homologue), enhances expression of the major immediate early promoter, whereas UL69

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In Utero Gene Therapy: Transfer and Long-Term Expression of the Bacterial *neo*^{r} Gene in Sheep after Direct Injection of Retroviral Vectors into Preimmune Fetuses

CHRISTOPHER D. PORADA,[1] NAM TRAN,[1] MARTIN EGLITIS,[2] ROBERT C. MOEN,[3] LISA TROUTMAN,[3] ALAN W. FLAKE,[4] YI ZHAO,[5] W. FRENCH ANDERSON,[5] and ESMAIL D. ZANJANI[1]

ABSTRACT

We investigated whether directly injecting retroviral vectors into preimmune fetuses could result in the transfer and long-term expression of exogenous genes. Twenty-nine preimmune sheep fetuses were injected with helper-free retroviral vector preparations. Twenty-two fetuses survived to term, 4 of which were sacrificed at birth. Of the remaining 18 animals, 3 were controls and 15 had received vector preparations. Twelve of these 15 animals demonstrated transduction of hematopoietic cells when blood and marrow were analyzed by *neo*^{r}- specific PCR. Eight experimental sheep have been followed for 5 years, during which time we have consistently observed proviral DNA and G418-resistant hematopoietic progenitors. The G418-resistant colonies were positive when analyzed by *neo*^{r}- specific PCR. *neo*^{r} gene expression was also demonstrated using several immunological and biochemical methods. The transduction of hematopoietic stem cells was confirmed when lambs transplanted with bone marrow from in *utero*-transduced sheep exhibited *neo*^{r} activity in marrow and blood. Vector distribution was widespread in primary animals without pathology. PCR analysis indicates that the germ line was not altered. These studies demonstrate that direct injection of an engineered retrovirus is a feasible means of safely delivering a foreign gene to a developing fetus and achieving long-term expression without modifying the germ line of the recipient.

OVERVIEW SUMMARY

Porada *et al.* describe a direct and relatively simple approach for *in utero* gene therapy by the demonstration of transfer and long-term expression of the *neo*^{r} gene in sheep after the direct intraperitoneal injection of the vector or producer cells into preimmune fetuses. The long-term expression of the exogenous gene without significant safety problems suggests that this procedure may represent a useful therapeutic approach for the early gene therapy of genetic diseases.

INTRODUCTION

Hematopoietic Stem Cell (HSC)-based gene therapy offers the promise of treating a wide array of genetic diseases including the hemoglobinopathies, storage diseases, and other metabolic disorders, and diseases of immune function such as adenosine deaminase (ADA) deficiency (Karlsson, 1991; Anderson, 1992; Miller, 1992). Postnatal gene therapy studies performed in murine models have demonstrated that it is possible to

[1] Departments of Veterans Affairs Medical Center, Reno, NV 89520.
[2] National Institutes of Health, Bethesda, MD 20892.
[3] Baxter Healthcare Corporation., Round Lake, IL 60073.
[4] Children's Hospital of Philadelphia, Philadelphia, PA 19104.
[5] Gene Therapy Laboratories, University of Southern California School of Medicine, Norris Cancer Center, Los Angeles, CA 90033.

transfer therapeutic genes successfully with high efficiency into putative HSC populations and achieve high-level multilineage expression of the transgenes following transplantation of the gene-modified HSCs into lethally irradiated recipients (Eglitis *et al.*, 1985; Correll *et al.*, 1992; Ohashi *et al.*, 1992; Plavec *et al.*, 1993; Schiffmann *et al.*, 1995). However, studies in larger animals and in clinical trials have shown that the efficiency of transduction of human/large-animals HSCs is considerably lower than that observed in the murine system (Ekhterae *et al.*, 1990; Bodine *et al.*, 1993; Van Beusechem *et al.*, 1993; Bienzle *et al.*, 1994; von Kalle *et al.*, 1994; Ward *et al.*, 1994; Xu *et al.*, 1994). Despite this low transduction efficiency, promising results and clinical improvement have been observed (Bank, 1996; Kohn, 1997). With advancements in vector design and transduction methodology as well as *in vitro* HSC manipulation, it is likely that postnatal HSC gene therapy will prove to be a powerful and precise therapeutic modality for the treatment of numerous human genetic disorders.

An alternative approach to existing gene therapy protocols would be to perform gene therapy before birth, *in utero*. Carrying out gene transfer early in gestation would enable the delivery of the therapeutic gene before the genetic deficit had significantly compromised the patient. In addition, several features of the developing fetus make it an ideal target for HSC-directed gene therapy. The high proliferative status of the fetus is one of these characteristics. Numerous studies have now provided evidence that a higher percentage of the HSC clones present within a fetus are actively cycling at any given point in time than would be in an adult counterpart (Clapp *et al.*, 199 1; Haneline *et al.*, 1996). For this reason, fetal HSCs should be more amenable to gene transfer with the existing generation of murine retroviral vectors, which require cell division for transduction to occur. In support of earlier sources of HSCs being well suited for retrovirus-based gene transfer are clinical studies performed by Kohn *et al.* (1995), in which neonates with ADA deficiency were transplanted with gene-modified autologous umbilical cord blood HSCs. These trials have thus far demonstrated gene transfer and expression in significantly higher percentages of circulating lymphocytes than in previous trials performed on older patients. Furthermore, experimental studies have demonstrated that more efficient gene transfer with retroviral vectors can occur in populations of hematopoietic progenitors from fetal donors than when compared with cell populations taken from adult donors (Kantoff *et al.*, 1989; Ekhterae *et al.*, 1990).

Another aspect of fetal development that suggests a potential benefit by performing HSC gene therapy *in utero* is the expansion of the hematopoietic system during gestation. Conceivably, if transduction of even small numbers of primitive HSCs could be accomplished early in gestation, expansion of transduced HSCs could lead to significant transgene activity. In this regard, we have previously reported the successful transfer and long-term expression of the bacterial *neo*^{r} gene in sheep following an *in utero* HSC transplantation/retroviral transduction protocol (Kantoff *et al.*, 1989; Ekhterae *et al.*, 1990). The procedure involved collecting peripheral blood from 110-dayold fetal sheep (term, 145 days) by exchange transfusion with maternal blood, exposing the mononuclear cells to the N2 retroviral vector overnight, and then reinfusing these gene-engineered cells into the fetus. After birth, the *neo*^{r} sequence was detected in marrow and blood of many of these animals by polymerase chain reaction (PCR), and many of the animals contained G418-resistant hematopoietic progenitors of erythroid (CFU-E, BFU-E), myeloid (CFU-GM), and mix (CFU-Mix) phenotype, suggesting that primitive HSCs may have been transduced. That HSCs were transduced was further substantiated by the continued presence of G418-resistant progenitors and PCR positivity in two animals that were observed for up to 43 and 59 months after birth (Kantoff *et al.*, 1989). These studies demonstrated than an *in utero* approach to gene therapy could result in the transfer and long-term expression of the vector-encoded genes with a significantly higher efficiency than had been reported with other large-animal models, and suggested that an *in utero* approach might be a more successful route toward achieving long-term correction of genetic disorders.

Since the removal of fetal blood and multiple manipulations of the fetus precluded performing this type of procedure on very young fetuses, we wished to investigate whether a procedure that did not require blood removal from the fetus would allow the gene transfer to be performed at an earlier point in gestation. In the

present studies, we evaluated whether the direct injection of vector into preimmune sheep fetuses was a safe method for achieving efficient transduction and long-term expression of the vector-encoded genes within the hematopoietic system of the treated sheep. In this regard, previous studies by Clapp *et al.* (1995a,b) demonstrated the efficacy of such an approach in rodents by the direct injection of the vector into fetal rat livers. To address whether directly injecting vector preparations into a large animal could result in efficient transduction of HSCs, we injected 57- to 67-day-old sheep fetuses intraperitoneally with retroviral vector preparations (supernatant, producer cells, or irradiated producer cells), waited until birth, and analyzed the sheep for the presence/expression of the *neo*^{r} gene. In 12 of the 15 sheep that received vector and survived to term, the *neo*^{r} gene was detected by PCR within the first 6 months of life. Expression of the *neo*^{r} gene was demonstrated by G418-resistant hematopoietic colony growth. In eight of these sheep, which have now been followed for more than 5 years, we have consistently observed the presence/expression of the *neo*^{r} gene as assessed by PCR and G418 resistance assays. In addition, expression of the *neo*^{r} gene was confirmed by (1) functional neomycin phosphotransferase (NPT) activity assay, (2) immunofluorescence, (3) enzyme-linked immunosorbent assay (ELISA), and (4) fluorescence-activated cell sorting (FACS) analysis. While the long-term presence/expression of the *neo*^{r} gene suggested the transduction of HSCs, this result was confirmed when lambs transplanted with HSCs from transduced sheep exhibited *neo*^{r} activity in bone marrow (BM) and peripheral blood (PB). PCR analysis of sacrificed primary animals revealed that the vector sequences were present in almost all tissues analyzed, including the reproductive organs. However, no pathology was noted in any of the organs. Furthermore, breeding experiments and PCR analysis of purified sperm cells from three rains indicate that the germ line was not altered. These studies demonstrate that directly injecting an engineered retrovirus is a safe and efficient way to deliver a foreign gene to a developing fetus and achieving long-term expression in hematopoietic cells without appearing to place the fetus or its germ line at risk.

MATERIALS AND METHODS

Gene transfer protocol

Three different retroviral vectors created with the Gl vector system were used in these studies: G1NaSvAd.24, G1BgSvNa.29, and G1TkSvNa.90 (Genetic Therapy, Gaithersburg, MD). All are Moloney murine leukemia virus (MoMuLV)-based and contain the *neo*^{r} gene. The GINaSvAd.24 vector contains the *neo*^{r} gene under the transcriptional control of the viral long terminal repeat (LTR), while in G1TkSvNa.90 and GlBgSvNa.29, the *neo*^{r} gene is driven by an internal simian virus 40 (SV40) promoter. These vectors were packaged in PA317 or Pat2.4 packaging lines, respectively, according to established procedures (Cornetta *et al.*, 1991). All vectors were free of replication-competent helper virus as determined by an extended sarcoma'/leukemia- (SI/L-) assay (Cornetta *et al.*, 1991). Supernatants containing one of the retroviral vectors, or its corresponding producer cell line, were injected into fetal sheep *in utero* as follows: pregnant sheep (57-67 days gestation; term, 145 days) were prepared for surgery with an injection of ketamine, and their abdomens were shaved and washed. Sheep were then anesthetized with a regulated endotracheal tube connected to a halothane tank, a midline incision was made, and the uterine horns were exposed. The fetus was visualized within the intact amnion, using the microbubble technique (Zanjani *et al.*, 1992), and an intraperitoneal injection of either 1-2 ml of retroviral supernatant (titers: GINaSvAd.24, I *X* 104; GlBgSvNa.29, 1.5 *X* 106) or of the producer cell lines (titers: PA317/GlNaSvAd, 2 X 105; Pat2.4/GlTkSvNa, 5 X 103) was given to each fetus, and the incisions were closed. The animals were allowed to come to term and deliver the lambs. After birth, peripheral blood and bone marrow were drawn at regular intervals for clonogenic assays and PCR analysis. During gestation, blood samples were drawn from the mothers and analyzed by PCR for the presence of the *neo*^{r} sequence. These tests were faintly positive in two cases (data not shown), in agreement with studies in mice (Tsukamoto *et al.*, 1995) demonstrating that the placental barrier allows the passage of liposome-coated DNA from mother to fetus, and, presumably, vice versa.

Detection of *neo*^{r} sequences in sheep tissue

Three hundred nanograms of total genomic DNA was subjected to analysis by PCR as described (Saiki *et al.*, 1985), with the following changes to the reaction constituents: primers used were 0.5 μM A (5' GGT GGA GAG GCT ATT CGG CTA TGA 3') and 0.5 *μM* B (5' ATC CTG ATC GAC AAG ACC GGC TTC 3'), which amplify a 440-base pair (bp) fragment of the bacterial *neo*^{r} gene. dATP, dCTP, and dGTP were used at 200 *μM*. To eliminate product carryover, dUTP (300 μM) was substituted for dTTP, and reactions were treated with uracil-DNA-glycosylase (UNG) prior to amplification (Wang *et al.*, 1992). MgC12 was used at 3.5 μM, and I unit of Amplitaq DNA polymerase was added to each 50-Al reaction (all PCR reagents were purchased from Perkin-Elmer, Norwalk, CT). The samples were overlaid with mineral oil, and 40 cycles of PCR performed as follows: 95°C for 1 min, 65°C for 1.5 min, and 72°C for 1.5 min. Fifteen microliters of each reaction was electrophoresed on a 2% agarose, Tris-borate-EDTA gel.

Southern blotting of PCR products

Southern blotting was performed essentially as previously described (Sambrook *et al.*, 1989). Briefly, after electrophoresis, the gel was transferred under denaturing conditions to Gene Screen Plus (Du Pont, Boston, MA). After transfer, the DNA was cross-linked to the membrane by short-wave ultraviolet (UV) irradiation. The filter was prehybridized at 65°C for 1 hr in 6 X SSC, 0.5% sodium dodecyl sulfate (SDS), and salmon sperm DNA (100 μg/ml); 2 X 10^6 cpm of random prime-labeled *neo*^{r} probe was added per milliliter of prehybridization solution, and the filter was allowed to hybridize for 4-6 hr at 65°C. The filter was washed four times at 65°C under conditions of increasing stringency and autoradiographed for 1-12 hr (depending on the signal intensity) at -70°C with two intensifying screens (Cronex Lightning Plus; Du Pont). The positive DNA consisted of varying amounts of the plasmid pUC18Neo (Genetic Therapy) mixed with DNA from normal sheep peripheral blood to yield 300 ng of total DNA per PCR reaction. The other peripheral blood and bone marrow DNA samples were prepared using a standard salting-out procedure. In short, 1 X 10^7 mononuclear cells were lysed in 0.5 *M* Tris (pH 9), 20 m*M* EDTA, 10 m*M* NaCl, 1% SDS, and proteinase K (600 μg/ml) and incubated overnight at 55°C. NaCl was then added to a final concentration of 1.5 *M*, the solution mixed, and proteins pelleted by centrifugation. DNA was then ethanol precipitated, collected by centrifugation, and resuspended in UV-irradiated, sterile H20.

RT-PCR

The expression of the *neo*^{r} gene was analyzed by reverse transcriptase (RT)-PCR, using RNA isolated from peripheral blood of the transduced sheep. Mononuclear cells were obtained by Ficoll-Hypaque (Sigma Chemicals, St. Louis, MO) density centrifugation, and total RNA was extracted using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD). RNA was subjected to DNase (GIBCO-BRL) digestion to ensure the absence of any contaminating genomic DNA prior to reverse transcription. RNA was reverse transcribed using the SuperScript preamplification system (GIBCO-BRL). One-tenth of the resultant cDNA was analyzed by *neo*^{r} -specific PCR and Southern blotting as described above.

Hematopoietic progenitor assays

The presence/activity of the *neo*^{r} gene in hematopoietic progenitors was assayed by testing hematopoietic cells for resistance to the neomycin-like antibiotic G418 (GIBCO-BRL). Bone marrow was obtained from sheep at regular intervals after birth, and mononuclear cells (2-5 X 10^5/ml) were cultured in plasma clot (CFU-E,

BFU-E) or methylcellulose (CFU-Mix, CFU-GM, BFU-E) as previously described (Roodman and Zanjani, 1979; Kantoff *et al.*, 1989). Bone marrow samples were aspirated into heparinized syringes from the posterior iliac crest of treated lambs and age-matched controls. Mononuclear cells were isolated by density gradient centrifugation over Ficoll-Hypaque (Sigma Chemicals), washed twice, and resuspended in Iscove's modified Dulbecco's medium (IMDM), 10% fetal calf serum (FCS) at desired concentrations. The cells were cultured in the presence or absence of different concentrations of G418 (2-3 mg/ml), erythropoietin (0.4 IU/ml), and sheep leukocytederived phytohemagglutinin (PHA)-stimulated leukocyte-conditioned medium (PHA-LCM) (5%, v/v). The plates were incubated at 37°C in a humidified atmosphere of 5% C02 in air for 4 days (CFU-E) or 9-12 days (CFU-GM and BFU-E). Hematopoietic colonies in plasma clot cultures were examined after transfer, fixing, and staining on glass slides (Roodman and Zanjani, 1979), while colonies in methylcellulose were enumerated *in situ* (Kantoff *et al.*, 1989), and the percentage of G418-resistant colonies calculated.

Gel-overlay assay for the expression of the neomycin phosphotransferase (neo^r) gene

The NPT gel-overlay assays were conducted as previously described (Reiss *et al.*, 1984). Bone marrow mononuclear cells (1 X 10^6) resuspended in 300 µl of phosphate-buffered saline (PBS) were freeze-thawed five times, and cellular debris was pelleted. The resultant supernatant was transferred to a new tube containing 300 µl of 2X sample buffer (0.2% SDS, 0.25 m*M* Tris-HCI [pH 6.8], 10% 2-mercaptoethanol [2-ME], 10% glycerol, 0.002% bromphenol blue). Thirty microliters of this sample was run on a nondenaturing 5% stacking/12% separating polyacrylamide gel in the Mini-Protean system (BioRad Laboratories, Hercules, CA). The gel was then processed as previously described to visualize NPT activity (Reiss *et al.*, 1984).

Immunofluorescence detection of neomycin phosphotransferase in peripheral blood smears

Immunofluorescence assays were performed according to standard procedures on peripheral blood smears of sheep. Smears of peripheral blood were prepared on glass slides and allowed to air dry overnight; they were then fixed in ice-cold methanol, washed with PBS, and blocked with bovine serum albumin (BSA) in PBS. Slides were washed twice with PBS, and incubated with a polyclonal antibody to NPT (5 Prime-3 Prime, Boulder, CO). After incubation, the slides were washed twice and the primary antibody detected with a fluorescein isothiocyanate (FITC)-labeled monoclonal antibody to rabbit IgG (Sigma Chemicals). The slides were then washed three times, and the nuclei counterstained with propidium iodide (Oncor, Gaithersburg, MD). The slides were viewed on a fluorescence microscope fitted with a dual filter cube allowing the simultaneous visualization of propidium iodide and FITC (Olympus America, Melville, NY). One thousand cells were counted in each sample to determine the percentage of cells expressing NPT. Negative controls consisted of peripheral blood smears obtained from three different normal control sheep. No staining was seen in any of the control sheep.

Transplantation of BMNCs obtained from transduced sheep to secondary fetal recipients

Bone marrow was aspirated from sheep 167, 177, and 182 at 2.5 years posttransduction, and mononuclear cells (BMNCs) isolated using Ficoll-Hypaque (Sigma Chemicals) density gradient centrifugation. Preinumme fetal sheep recipients (58-62 days old) were transplanted as follows: 5 fetuses received 30 X 10^6 cells/fetus from animal 167, 2 fetuses were given 30 X 10^6 cells/fetus from animal 177, and 10 fetuses were transplanted with 20 X 10^6 cells/fetus from animal 182. At birth, and at intervals thereafter, bone marrow and peripheral blood were drawn from these secondary recipients, and G418-resistance progenitor assays and *neo^r* -specific PCR were performed to assess the presence and expression of the vector-encoded genes within cells of the various hematopoietic lineages in these recipients.

Fluorescence in situ *hybridization*

As a further means of evaluating tissue distribution of the provirus, animal 199 was sacrified at 20 months posttransduction and its organs collected. The organs were washed extensively with PBS, and were subsequently fixed for 48 hr in a 10% buffered formalin solution. The organs were then paraffin embedded, sectioned, and mounted on silanized glass slides. Fluorescence *in situ* hybridization (FISH) was performed using a commercially available kit according to manufacturer instructions (Oncor). The probe utilized consisted of the plasmid pUC18Neo (Genetic Therapy) random-prime labeled with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN). After hybridization, detection, and counterstaining, the slides were viewed on a fluorescence microscope fitted with a dual filter cube allowing the simultaneous visualization of propidium iodide and FITC (Olympus America).

ELISA for neomycin phosphotransferase

Mononuclear cells (10^7) obtained by Ficoll-Hypaque density gradient separation from blood of primary transduced sheep at 50-52 months posttransduction were transferred to 1.5-ml microcentrifuge tubes, washed three times with 1 ml of PBS, resuspended in 1 ml of PBS containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and lysed by repeated freeze (liquid N2)-thaw (37°C H20 bath) cycles. After the removal of cellular debris, 200 µl of each cell extract was analyzed by NPT ELISA, using a commercially available kit according to the manufacturer instructions (5 Prime-3 Prime). Negative controls consisted of mononuclear cells from three different normal control sheep. Purified NPT used to generate the standard curve was provided by the manufacturer.

FACS analysis of peripheral blood to detect neomycin phosphotransferase expression

Peripheral blood mononuclear cells (1 X 10^6 from each sheep) were transferred to separate tubes and pelleted at 350 X *g* for 8 min. The pellets were resuspended in cold PBS, 0.1% sodium azide. Cells were fixed in 2% paraformaldehyde, and their membranes permeabilized with 0.05% Tween 20. The cells were blocked with normal goat serum, and stained with a commercially available polyclonal antibody to NPT (5 Prime-3 Prime). NPT was visualized with a FITC-labeled monoclonal antibody to rabbit IgG (Sigma Chemicals), and analyzed using a FACScan flow cytometer (Becton Dickinson Immuno-Systems, San Jose, CA). Negative controls consisted of peripheral blood cells from a normal control sheep stained by the same protocol as the experimental animals.

RESULTS

Experimental design

The overall design and outcome of these studies are summarized in Table 1. A total of 29 preimmune fetal sheep was injected with either retroviral supernatant, producer cells, or empty packaging cells; 22 survived to term. Two of the seven recipients that died prior to birth (animals 184 and 185) did so as a result of the pregnant ewe being struck by the feed truck,

TABLE 1. EXPERIMENTAL SUMMARY[a]

Animal No.	*Injected with:*	*Outcome*	NeoR
activity			
167	1 ml Pat2.4/G1TkSvNa.90 cells	Sac. at 3.5 years of age	Positive
168	1 ml Pat2.4/G1TkSvNa.90 cells	Sac. at birth	nd
178	2 ml Pat2.4/G1TkSvNa.90 cells	Sac. at 1 month of age	nd
179	2 ml Pat2.4/G1TkSvNa.90 cells	Alive	Positive
180	1 ml Pa317/G1NaSvAd.24 cells	Sac. at birth[b]	nd
181	1 ml Pa317/G1NaSvAd.24 cells	Sac. at birth[b]	nd
183	1 ml Pa317/G1NaSvAd.24 cells	Sac. at 4 months of age	nd
172	2 ml irradiated Pat2.4/G1TkSvNa.90 cells	Sac. at 7 months of age	nd
173	2 ml irradiated Pat2.4/G1TkSvNa.90 cells	Alive	positive
174	2 ml irradiated Pat2.4/G1TkSvNa.90 cells	Absorbed	nd
175	2 ml irradiated Pat2.4/G1TkSvNa.90 cells	Sac. at 7 months of age	Positive
186	1 ml irradiated Pa317/G1NaSvAd.24 cells	Died at 3 months of age[c]	Positive
187	1 ml irradiated Pa317/G1NaSvAd.24 cells	Sac. at 10 months of age	Positive
188	1 ml irradiated Pa317/G1NaSvAd.24 cells	Absorbed	nd
176	2 ml G1NaSvAd.24 supernatant	Alive	Positive
177	2 ml G1NaSvAd.24 supernatant	Alive	Positive
182	2 ml G1NaSvAd.24 supernatant	Alive	Positive
189	2 ml G1NaSvAd.24 supernatant	Sac. at 4.5 years of age	Positive
184	2 ml G1BgSvNa.29 supernatant	Died 2 months postinjection[d]	nd
185	2 ml G1BgSvNa.29 supernatant	Died 2 months postinjection[d]	nd
198	2 ml G1BgSvNa.29 supernatant	Alive	Positive
199	2 ml G1BgSvNa.29 supernatant	Sac. at 1.2 years of age	Positive
190	2 ml Pat2.4 control cells	Absorbed	nd
200	2 ml Pat2.4 control cells	Absorbed	nd
201	2 ml Pat2.4 control cells	Absorbed	nd
194	1 ml Pa317 control cells	Alive	Negative
195	1 ml Pa317 control cells	Died at 3 months of age[e]	nd
196	1 ml Pa317 control cells	Sac. at 7 months of age	Negative
197	1 ml Pa317 control cells	Sac. at birth	nd

[a]Summarized here is the experimental design employed in these studies, the treatment given to each of the sheep, and the outcome of the manipulation, i.e., whether the animal survived to term. Most fetal loss occurred in the control group, with three of seven animals absorbing *in utero,* and one animal that died at 3 months of age with multiple abscesses in the lung and liver. Also presented here are the results of various tests to determine whether the animal contained and expressed the vector-encoded genes. If any of the methods employed in these studies detected the *neo*^{r} gene, then the sheep is marked as positive.

[b]Difficult delivery; newborn sacrificed.

[c]Caught in fence.

[d]Mother carrying twins was struck by feed truck.

[e]Died with multiple abscesses in liver and lungs.

Abbreviation: nd, Not determined; sac., sacrificed.

so that only five animals died as a possible result of the experimental procedure. Three had received empty packaging cells alone (no vector present). Although the reasons for such a significant fetal loss in this group could not be determined, it is clear that these losses were not due to the vectors used for gene transfer.

Of the 22 animals that survived to term, 2 (animals 180 and 181) were sacrified at birth owing to difficulty in delivery, and 2 others (animals 168 and 197) were sacrified at birth for tissue distribution analysis, leaving 18 animals for studies of gene transfer efficiency; 3 of these (animals 194, 195, and 196) were controls that received empty packaging cells. Bone marrow and blood from all animals were analyzed for the presence of vector DNA by PCR using primers specific for the *neo*^{r} gene shortly after birth and at intervals thereafter. None of the three animals receiving packaging cells alone exhibited *neo*^{r} activity at any time by PCR or G418resistance studies. Lamb 195 died at 3 months of age with multiple abscesses in the liver and lung, and lamb 196 was sacrified at 7 months; animal 194 has been kept alive for observation. Of the remaining 15 lambs, 12 were found to be positive in both marrow and blood. Results from 11 of the lambs exhibiting *neo*^{r} activity, including 6 animals that were given supernatant preparations, 2 animals that received producer cells, and 3 animals that were injected with irradiated producer cells, are shown in Fig. 1. Of these, five lambs that received a supernatant preparation (animals 176, 177, 182, 189, and 198), two lambs given producer cells (animals 179 and 167), and one lamb injected with irradiated producer cells (animal 173) were selected for long-term evaluation of both gene transfer efficiency and expression of the exogenous genes, while the remainder of the experimental animals were sacrified at various time points during the course of these studies to assess proviral tissue distribution. The eight sheep selected for long-term observations were evaluated for the presence/expression of *neo*^{r} by a variety of techniques.

G418 resistance studies

The efficiency of gene transfer into hematopoietic cells in all animals was determined by the ability of hematopoietic progenitors to form colonies in the presence of lethal concentrations of G418 *in vitro*. As we have reported (Kantoff *et al.*, 1989; Ekheterae *et al.*, 1990) and is also shown in Fig. 2, the formation of CFU-Mix-, CFU-GM-, and BFU-E/CFU-E-derived colonies by sheep bone marrow cells was inhibited by G418 in a dose-dependent fashion. These data were collected 16 months posttransfer. When compared with cells from untreated animals, colony growth by bone marrow of animals receiving either supernatant or producer cells was normal in number, morphology, and size in the absence of G418 selection. At 2 mg of G418 per milliliter, a small number of colonies was observed in the cultures from untreated sheep and from the sheep receiving only the packaging cell lines, but none were seen at concentrations >2 mg/ml. In contrast, significant numbers of colonies were detected in the presence of >2 mg of G418 per milliliter in cultures derived from bone marrow of sheep receiving either supernatant or producer cells (Fig. 2A). When compared as a group, animals receiving producer cells exhibited a higher level of G418 resistance in their marrow progenitors (Fig. 2B).

To ensure that colonies growing in G418 were doing so because they had been transduced, resistant colonies were removed from the methylcellulose plate and subjected to *neo*^{r} specific PCR analysis. As can be seen in Fig. 3, the picked G418-resistant colonies contained the *neo*^{r} gene, indicating that the retroviral vector had indeed integrated into their genomes, and was responsible for their ability to grow in G418.

To determine further whether G418 resistance of progenitors was an accurate indication of expression of the transgene, bone marrow was drawn from animal 167 at 18 months posttransduction, and clonogenic assays were conducted in the presence/absence of G418 (3 mg/ml) and/or 6 μM ganciclovir. Animal 167 had received the G 1 TkSvNa producer cell line, which produces vector encoding the herpes simplex thymidine kinase gene in addition to the *neo*^{r} gene. Cells expressing the genes carried within this vector should express thymidine kinase and be sensitive to ganciclovir, while untransduced cells should not. Results presented in Fig. 4 demonstrate that essentially all of the colonies from animal 167 that were resistant to G418 were also sensitive to 6 μM

ganciclovir, demonstrating that the growth of these colonies in G418 was likely due to the expression of the vector-encoded genes, and that both vector-encoded gene products were being expressed in the same colonies.

Neomycin phosphotransferase studies

Expression of the *neo*^{r} transgene in hematopoietic cells *in vivo* was evaluated by the presence of NPT activity in bone marrow and peripheral blood mononuclear cells. Since the *neo*^{r} gene product possesses the ability to transfer a terminal phosphate group from a donor molecule to kanamycin, we assayed proteins extracted from marrow mononuclear cells for their ability to transfer a radiolabeled phosphate from [γ-32P] ATP to kanamycin, using an established gel overlay assay. We were able to detect phosphotransferase activity at 20 months post transduction in four of the six transduced sheep that were tested by this relatively insensitive assay (Fig. 5), which was unable to detect NPT activity in animals 167 and 179, both of which exhibited *neo*^{r} activity on several occasions using other methods.

Results presented in Fig. 6 demonstrate NPT activity in peripheral blood of treated animals at 3 years posttransduction as determined by immunofluorescence using a commercially available antibody to NPT. Shown in Fig. 6 are the levels ofexpression within six animals, expressed as the percentage of total numbers of white blood cells that stained positive. As can be seen, detectable levels of NPT expression were observed in the peripheral blood of all six sheep at 3 years posttransduction. In some animals, the level of circulating white cells that expressed the gene was as high as 2.8%. The only cells that were counted positive were those that presented an intense cytoplasmic staining. Little or no staining was seen in any of three normal sheep and animal 194. Shown in Color Plate 1 are photographs of a representative blood smear from a control animal (Color Plate 1A) and from animal 182 (Color Plate 1B). As can be seen, there are a considerable number of positive cells (stained green) within the smear of animal 182, while there is no significant staining within the control cells (red stain is propidium iodide counterstain, which binds to all DNA). The intensity of the fluorescence also suggests that the level of expression within the cells from animal 182 may be significant.

The expression of the *neo*^{r} transgene was also measured by ELISA and flow cytometry. The ELISA was performed on peripheral blood mononuclear cells from seven sheep at 50-52 months posttransduction, using a commercially available ELISA kit specific for NPT. Shown in Fig. 7 are the highest values that were obtained from these animals during four different samplings over a 2-month period. As can be seen, six of the seven sheep analyzed at this time point contained a significant level of NPT, with animals 177 and 182 exhibiting the highest levels among the group. This variability in expression cannot be attributed to a difference in the vector preparation received, since four of the seven animals tested received G1NaSvAd supernatant. Of note, however, is sheep 198 (which received G1BgSvNa.29 supernatant), because this animal was the only one tested that exhibited no expression at all, suggesting that this vector may not yield as high a level of transduction and/or expression as the G1NaSvAd vector.

Results presented in Table 2 and Fig. 8 demonstrate that the vector-encoded *neo*^{r} gene is being expressed within a significant percentage of the hematopoietic cells in the circulation. Flow cytometric analysis of Ficoll-Hypaque-separated blood mononuclear cells obtained 55 months posttransfer using an NPT-specific antibody revealed the presence of NPT-positive cells within the ungated (0-4.85%), the lymphocytic (0-1.59%), and the granulocytic/monocytic (0-13.1%) populations.

Assessment of proviral integration by PCR

The animals were also monitored at intervals after birth for the presence of proviral DNA by using PCR with *neo*^{r} primers. Overall, during the course of these studies, bone marrow from many of the sheep that received vector

was positive at the majority of sampling points, while the *neo*^{r} signal was seen only intermittently in the peripheral blood of these animals. This consistent positivity in marrow is demonstrated in Fig. 9, which shows the results of a PCR performed on bone marrow samples obtained from animal 182 at various time points post-transduction. As can be seen, at the majority of sampling times, we were able to detect the *neo*^{r} gene, demonstrating the longterm persistence of transduced cells within the hematopoietic compartment of this animal. The long-term persistence of *neo*^{r}-positive cells within marrow and their expression within peripheral blood cells suggest that successful transduction of the primitive hematopoietic stem cells (HSCs) may have occurred in these animals.

Transplantation of BMNCs from in utero-*transduced animals to assess transduction of HSCs*

We used the competitive engrafting *in utero* HSC transplantation approach (Zanjani *et al.*, 1992) to assess the presence of transduced long-term engrafting HSCs in bone marrow of treated sheep. In this model of competitive HSC engraftment, engrafted donor HSCs persist for long periods (>7 years) and undergo multilineage differentiation. Bone marrow mononuclear cells were obtained from sheep 167, 177, and 182 at 2.5 years posttransduction and transplanted into 17 preimmune secondary fetal sheep recipients as detailed in Materials and Methods. At intervals after birth, peripheral blood and bone marrow from the sheep were analyzed by *neo*^{r} -specific PCR. Figure 10 shows a representative PCR from these animals, in which 9 of the 12 secondary recipients analyzed contained the proviral DNA within their bone marrow, strongly supporting the transduction of primitive HSCs in the donor sheep. To evaluate the expression of the *neo*^{r} gene, methylcellulose and plasma clot cultures were performed on BMNCs from these secondary recipients at 15 months posttransplant, and, as can be seen in Table 3, five of the animals contained G418-resistant progenitors of erythroid and myeloid origin, further supporting that transduction of primitive HSCs capable of engraftment and multilineage expression had occurred as a result of the direct *in utero* injection of retroviral vectors.

Analysis of tissue distribution of vector

To determine whether retroviral transduction *in vivo* was limited to cells of hematopoietic origin, or whether other tissues of the fetus had also been transduced, DNA was extracted from different tissues of two sheep that were sacrificed at birth. *neo*^{r}-specific PCR was performed, followed by Southern blotting to confirm the identity of the products. As can be seen in Fig. 11, all of the tissues from this representative animal (168) contained the *neo*^{r} sequence, indicating that transduction may not have been limited to cells of hematopoietic origin. Unfortunately, bone marrow and peripheral blood were unavailable for analysis, and are therefore not shown. To determine whether proviral DNA persisted in cells of nonhematopoietic organs, animal 187 was sacrificed 14 months after gene transfer. DNA was extracted from lung, liver, kidney, and testes of this animal and analyzed by *neo*^{r} PCR. The results of this analysis are presented in Fig. 12. Provirus was detected in the bone marrow, kidney, and testes of sheep 187. The persistence of proviral DNA in brain tissue of animal 199 at 1.2 years posttransduction was demonstrated by PCR (Color Plate 2A) and fluorescence *in situ* hybridization (Color Plate 2B). In all cases pathologic examination revealed no abnormalities in any organs examined. Although these findings strongly suggest proviral integration into genomes of nonhematopoietic cells of these animals, we have not yet performed immunohistochemistry on these tissues to determine which cell types within the organs contain/express the vector-encoded *neo*^{r} gene. It is also important to note that the tissues were not exhaustively perfused to rid the tissue of contaminating blood cells and macrophages. In this regard, the following results suggest that the positive PCR signal in the testes did not signify the transduction of the germ cells.

Heritability of the vector-encoded genes

The fact that the provirus was detected within the testes of animal 187 suggested that the germ cells may have been transduced *in vivo*, potentially enabling the vector-encoded genes to be passed on to subsequent offspring. We bred sheep 182 (a ram) with four normal control ewes, and allowed one of the ewes to lamb normally, while the other fetuses were sacrificed *in utero* and their tissues collected. There were five resultant offspring (the normal birth is twins). We sacrificed four of the animals to examine the DNA from all of the tissues, since the provirus should be present within all the tissues of the body if the father's germ cells were indeed transduced. DNA was extracted from the brain, kidney, liver, lung, spleen, pancreas, thymus, and reproductive organs, and *neo*^{r}-specific PCR was performed. The results shown in Fig. 13 (a representative PCR from one of the offspring) demonstrate that the provirus was absent in all of the organs tested from all four of the sacrificed animals, suggesting that high-efficiency transduction of the germ cells of animal 182 had not occurred.

Absence of neo^r *in sperm cells*

To test more directly whether the germ line had been transduced in these animals, ejaculates were collected from animal 182 and analyzed by PCR. As can be seen in Fig. 14a, the ejaculate (semen) from this animal was occasionally positive when analyzed by *neo*^{r}-specific PCR. To rule out the possibility that this positivity was due to contamination of the ejaculate with cells other than sperm cells, i.e., white blood cells, an ejaculate from this same ram was divided into two populations, one consisting of the total ejaculate, and, from the other half of the ejaculate, a pure population of sperm cells was isolated using a Percoll density gradient centrifugation method. Isolated sperm cells were then subjected to *neo*^{r}-specific PCR, as was the total ejaculate. As is also shown in Fig. 14a, these pure populations of sperm cells were consistently negative for the *neo*^{r} sequence, suggesting that the presence of the proviral DNA within the complete ejaculate from animal 182 was likely due to contamination of the ejaculate with cells of nonsperm origin. Further proof for this lack of transduction of true sperm cells came from a *neo*^{r}-specific PCR performed on purified sperm cells from the remaining rams in this study. As can be seen in Fig. 14b, purified sperm cells from rams 167, 176, and 182 were consistently negative for the *neo*^{r} gene.

DISCUSSION

The results of the studies presented here demonstrate that the direct intraperitoneal injection of retroviral vectors or producer cells into preimmune sheep fetuses results in the transfer and long-term expression of the vector-encoded genes within the hematopoietic cells of these animals, as evidenced by the presence of G418-resistant hematopoietic: progenitors in the bone marrow of these animals for several months after birth, the presence of proviral DNA in marrow and blood, and the presence of NPT activity in peripheral blood and marrow. This expression has been long term, persisting throughout the 5-year course of these studies. The long-term presence/expression of the vector-encoded genes in the peripheral blood and marrow of these animals suggests that transduction of primitive, repopulating HSCs had occurred following the direct intraperitoneal vector injection. This conclusion was confirmed by competitive secondary transplantation, in which bone marrow mononuclear cells taken from the primary animals were transplanted into secondary preimmune fetal sheep recipients and proviral DNA and NPT activity were detected in peripheral blood and marrow of the secondary recipients at 6 months posttransplant.

These results also demonstrate that the transfer of the exogenous genes was not limited to the hematopoietic cells, but occurred in other tissues as well, including the reproductive organs (testes). Importantly, the presence of these genes in other tissues produced no detectable pathology, and we did not observe any toxicity or problems associated with this procedure. Although we did experience some early losses of animals, most occurred in the control group, implying that the vector preparations were not the causative agent. Furthermore, the presence of provirus within the reproductive organs was not the result of transduction of the germ line, since the exogenous genes were not transferred to offspring in the breeding experiments performed, and purified sperm cells were devoid of proviral DNA when analyzed by PCR.

Also of note is the transfer of exogenous genetic material into the material circulation following intraperitoneal injection of the fetus. Interestingly, three ewes were found to contain, transiently, trace quantities of vector sequences in their peripheral blood (data not shown), and these ewes were carrying fetuses that were injected with producer cell lines. None of the ewes whose fetuses received vector-containing supernatant were found to be positive for vector sequences in their circulation, raising the possibility that the faint signals obtained in the three positive ewes was somehow due to the injection of retroviral vector producer cells into the fetuses, with subsequent passage of trace amounts of vector or cells through the placenta. This conclusion would be in agreement with other studies demonstrating that placental tissue is readily infected with a variety of retroviruses (Ueno *et al.*, 1983; McGann et al., 1994; Bui et al., 1995), and that the placenta allows the transfer of retroviral-like particles from mother to fetus during pregnancy (Tsukamoto et al., 1995). However, in our present studies, the transfer of genetic material that occurred was not long lasting, since it was detected only at one time point in each ewe, with all subsequent samplings being devoid of vector sequences. On the basis of the transient nature of the transfer, it is perhaps More likely that the appearance of vector sequences within the circulation of the mother was simply a result of fetal microhemorrhaging, which can occur during gestation and enables fetal blood cells to invade, transiently, the material circulation. In any case, the procedure employed in these studies does not seem to place the mother at any risk of permanent genetic alteration.

It is also interesting to note that the exogenous genes were detected in the brain of several animals that were sacrificed at various time points throughout these studies. It is not known whether the presence of proviral DNA in the brain is a result of the entry of the vector into the central nervous system, with subsequent transduction of nervous tissue, since it is also possible that the presence of vector sequences within the brain may reflect the migration of transduced hematopoietic microglial precursors into the brain during development. Regardless of the mechanism, however, the ability to delivery exogenous genes to the brain in utero offers the possibility of treating a number of patients with storage diseases that affect the central nervous system.

Observations in primary and secondary sheep recipients strongly suggest that transduction of long-term engrafting HSCs had occurred. This conclusion was demonstrated by the persistence of transduced hematopoietic cells and progenitors in primary recipients for several years, and by the long-term presence in secondary hosts following in utero transplantation of BMNCs from the in utero-transduced animals. A number of attempts have been made to carry out in utero gene transfer in large-animal models, including sheep, using adenoviral (Simon et al., 1993; Zabner et al., 1994; McCray et al., 1995; Vincent et al., 1995) or retroviral (Pitt *et al.*, 1995) vectors. While successful gene transfer was achieved, the investigators reported the development of potent cellular-mediated immune responses that eliminated both the vector and the vector-containing cells, resulting in only transient transgene expression. However, in all cases, the treatments were initiated late in gestation at a time when the fetuses were capable of mounting an immune response. The successful long-term gene transfer and expression reported here are likely the result of the preimmune status of the early gestational fetuses employed in these studies. There is a period in early immunologic development, prior to thymic processing of mature lymphocytes, during which the fetus is tolerant of foreign antigens. Exposure to antigen during this period results in sustained tolerance that can be permanent if the presence of the antigen is maintained (Billingham *et al.*, 1956; Binns, 1967). Cellular tolerance appears to be secondary to clonal deletion of reactive lymphocytes in the thymus, whereas the mechanism of B lymphocyte tolerance (peripheral tolerance) appears to involve both clonal deletion and clonal suppression (Marrack *et al.*, 1988; Schwartz, 1989; Adams, 1990). The end result is an immune system that is specifically tolerant of foreign antigenic sources. The use of preimmune fetuses and an intraperitoneal route of administration may have also circumvented the rapid inactivation of murine retroviral vectors that has been reported to occur *in vivo* (Takeuchi *et al.*, 1994; Rother *et al.*, 1995; Shimizu *et al.*, 1995).

While analyzing the animals in these studies, we observed that animals injected *in utero* with producer cells exhibited a higher level of transduction and G418 resistance than supernatant-treated animals. The possibility exhists that transduction is more efficient following release of vector particles *in situ* near their target cells, and that the slow continuous release of vector particles exposes the target cells to the vector over the course of days for a more efficient transduction. On the basis of the kinetics of HSC cycling, the availability of vector over long periods should ensure that larger numbers of HSC clones undergo cell cycle in the presence of vector, and are thus amenable to retroviral transduction. Another possibility that is currently under investigation in our laboratory is that the injected producer cells persist and proliferate in these animals, potentially producing vector particles for extended periods of time *in vivo*.

In theory, one advantage to performing gene therapy in a fetal setting is that, because of the naturally occurring expansion of the fetal hematopoietic system, transduction of relatively few HSCs could lead to a significant level of transgene activity. However, in the best of our animals, we detected only about 5% transduced cells in peripheral blood, which does not suggest that a large degree of expansion occurred. In the marrow, however, we detected much higher levels of transduction, as assessed by G418 resistance progenitor assays. It is difficult to reconcile this difference between marrow and blood, but it is interesting to note that others have observed a similar pattern of a high percentage of transgene-expressing cells in marrow and relatively low activity in peripheral blood (Kohn *et al.*, 1995). In our studies, it could be argued that this discrepancy was perhaps due to the fact that in this system, the transduced cells have no selective advantage. However, previous studies in human ADA-deficient patients have obtained similar results (Kohn *et al.*, 1995), suggesting that this dichotomy may persist even when the transduced cells would be predicted to have a selective advantage over the host's own deficient cells. It is possible that this discrepancy is a reflection of the process of clonal succession that has previously been described in mice containing retrovirally marked stem cell populations (Lemischka *et al.*, 1986), such that only a small percentage of the transduced HSC clones is giving rise to mature hematopoietic cells at any given time *in vivo*. Since methylcellulose culture requires the presence of growth factors in order to achieve colony formation, perhaps this culture system does not accurately represent the number of transduced mature cells being generated *in vivo*. Clarification of the physiologic mechanisms responsible for this phenomenon will require a more thorough understanding of the hematopoietic process. Nevertheless, it is important to note that in such a large animal, the presence of 5%-transduced cells reflects an enormous number of cells.

In conclusion, the direct intraperitoneal injection of retroviral vectors or producer cell lines into early-gestational sheep fetuses results in transduction of HSCs and their progeny, and expression of the transgenes is long term, persisting for more than 5 years posttransfer. These studies also demonstrate that the transferred genes appear to enter both hematopoietic and nonhematopoietic tissues, suggesting that this approach to gene therapy may prove useful in diseases that are unrelated to hematopoiesis. While levels of transduction into hematopoietic cells may be below those required to provide therapeutic benefit, preliminary studies currently underway in our laboratory suggest that levels of transduction can be greatly improved if higher titer vectors are employed. We are also evaluating whether providing multiple injections of vector preparation to the fetus may further enhance the levels of transduction. With the development of envelope recombinant vectors that enable tissue-specific transduction, this technique should enable the safe delivery of therapeutic genes to a variety of tissues early in gestation, perhaps preventing the extensive damage that takes place during development of fetuses with certain genetic diseases.

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Address reprint requests to:
Dr. Esmail D. Zanjani
VA. Medical Center (151B)
1000 Locust Street
Reno, NV 89520

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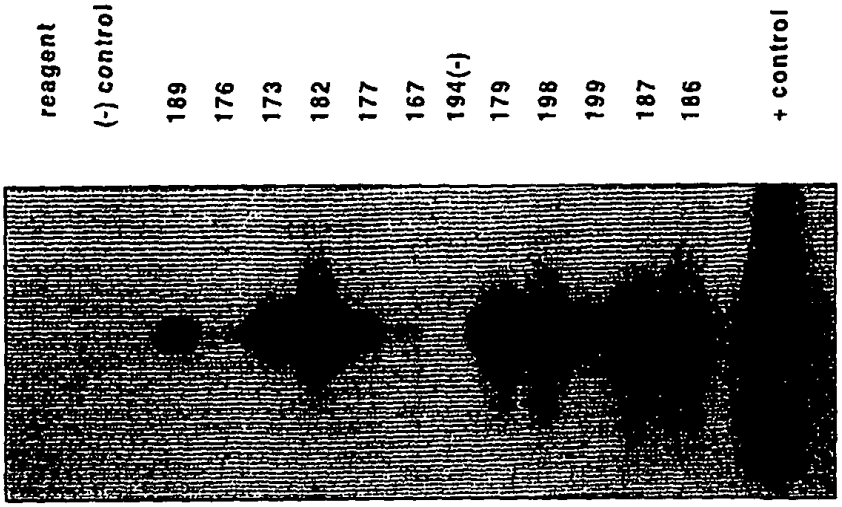


FIG. 1. Presence of *neo*^{r} gene in peripheral blood of *in utero*-transduced sheep at 6 months posttransduction. DNA was obtained from mononuclear cells isolated from peripheral blood by Ficoll-Hypaque density centrifugation. These DNA samples were then analyzed for the presence of the *neo*^{r} sequence by polymerase chain reaction. The reagent control consisted of all of the constituents of the PCR reaction mixture except template DNA. The negative control (–) DNA was isolated from the peripheral blood mononuclear cells of a normal control ram. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to a concentration of 10%. The remainder of the samples are marked.

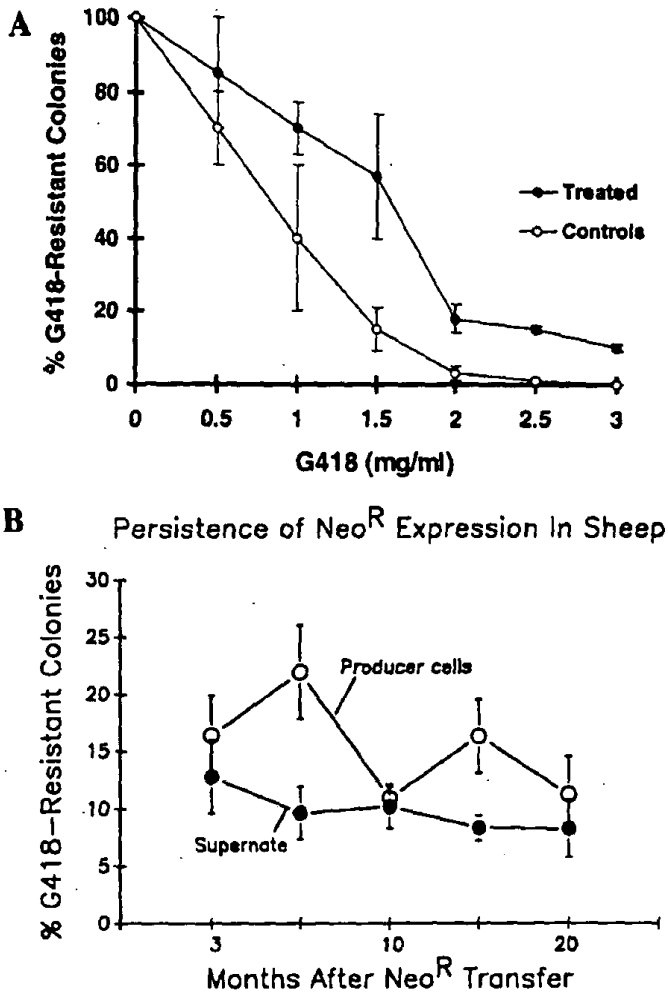


FIG. 2. G418 resistance of hematopoietic progenitors from *in utero*-transduced sheep. Mononuclear cells were obtained from bone marrow of the *in utero*-transduced sheep and grown in methylcellulose to determine the percentage of progenitors of myeloid, mix, and erythroid lineages that were resistant to increasing concentrations of G418. (A) The control values are the average of the percentage of colonies of each type that were resistant to each dose of G418. The values labeled "treated" are the average percentage of the combined colonies from sheep 167, 173, 176, 177, 179, and 182 that were resistant to each dose of G418. (B) Comparison of gene transfer efficiency with producer cell lines versus supernatant preparations. Values for each group represent the average G418 resistance of CFU-GM, CFU-Mix, CFU-E, and BFU-E from all of the animals in the study that received either producer cells or a supernatant preparation.

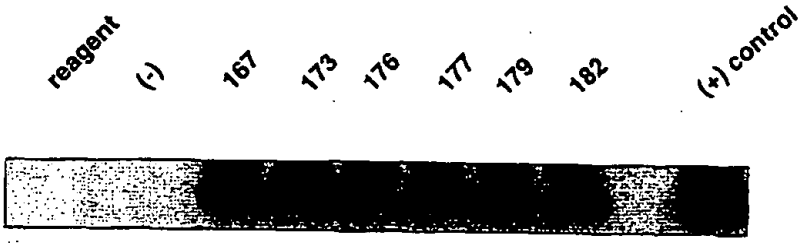


FIG. 3. Detection of *neo*^{r} sequence in DNA isolated from G418-resistant bone marrow colonies from *in utero*-transduced lambs, using the polymerase chain reaction. The negative control (–) DNA was isolated from colonies grown from bone marrow of a normal ram in the absence of G418; the reagent control represents all the constituents of the PCR reaction mixture except template DNA. The remaining samples are DNA isolated from G418-resistant methylcellulose colonies from transduced lambs, as indicated. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 10%.

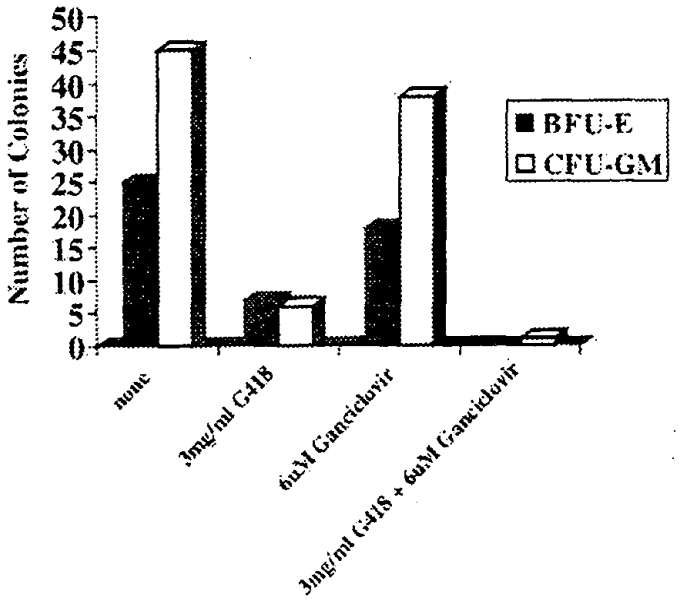


FIG. 4. Demonstration that G418 resistance of hematopoietic colonies is an accurate indication of vector-encoded gene expression (see text for details). CFU-GM and BFU-E colonies were grown from lamb 167 at 16 months posttransduction. Lamb 167 had received G1TkSvNa, a vector containing both the *neo*^{r} gene and the HSV thymidine kinase (*tk*) gene. All of the BFU-E and CFU-GM colonies from animal 167 that were resistant to G418 at 3 mg/ml were also sensitive to 6 μM ganciclovir, such that essentially no colonies were detected in the presence of both reagents.

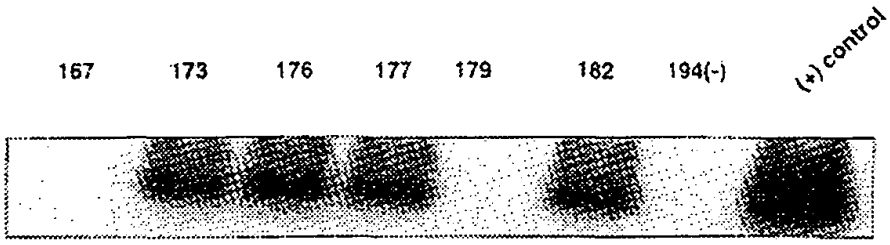


FIG. 5. Detection of neomycin phosphotransferase activity in bone marrow mononuclear cells from *in utero*-transduced lambs, using a gel overlay assay. Mononuclear cells were isolated from total bone marrow by Ficoll-Hypaque density centrifugation, and proteins were extracted under nondenaturing conditions as described in Materials and Methods. Samples were then run on a native polyacrylamide gel and assessed for their ability to transfer a ^{32}P-labeled phosphate from [γ-^{32}P]ATP to kanamycin, a substrate of neomycin phosphotransferase, as described in Materials and Methods. Transfer was detected by autoradiography. The positive control consisted of proteins isolated from *Escherichia coli* that had been transfected with a plasmid containing the *neo*^{r} gene.

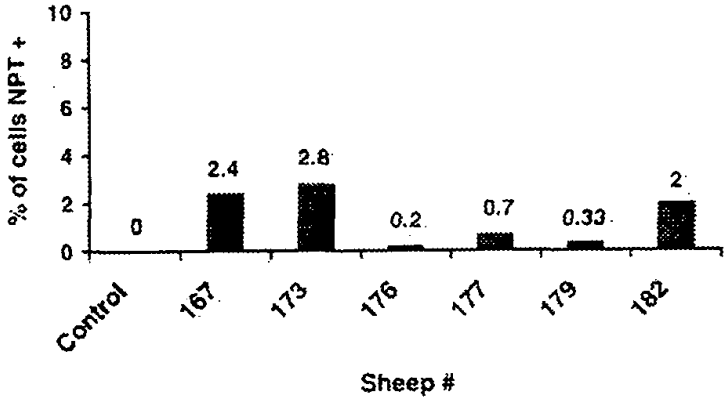


FIG. 6. Detection of NPT activity in peripheral blood smears from *in utero*-transduced sheep at 36 months posttransduction. Peripheral blood smears were prepared at 3 years posttransduction from sheep that had been transduced *in utero*, and these smears were then stained with an antibody specific for neomycin phosphotransferase (NPT). Shown here are the percentage of white blood cells (WBCs) in each smear that expressed NPT. The percentage of positive cells was determined by counting 1000 WBCs in each smear.

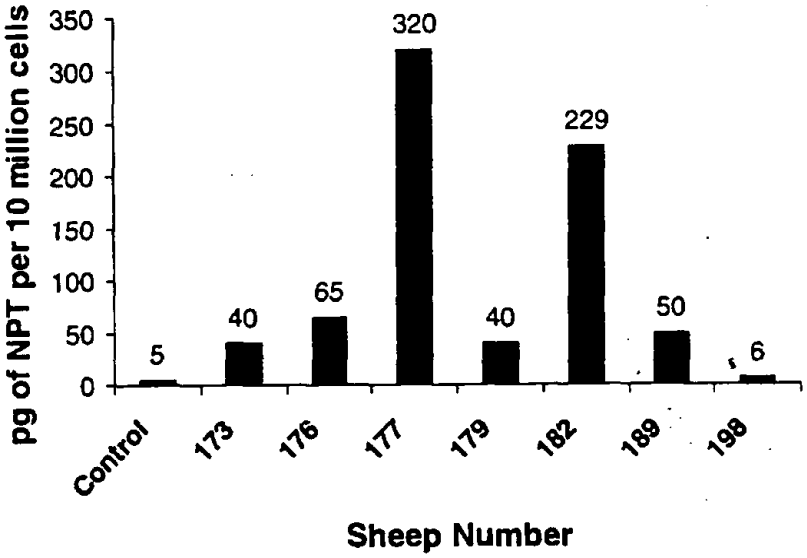


FIG. 7. NPT activity in PBMCs as detected by ELISA. Mononuclear cells (10^7) were collected from the peripheral blood of each of the experimental sheep at 50–52 months post-transduction and analyzed using an NPT-specific ELISA. Three control sheep were used to ensure that the assay was specific. All three control sheep consistently yielded low levels of background (<5 pg/10^7 cells), and the standard curve obtained with a dilution series of purified NPT was always linear.

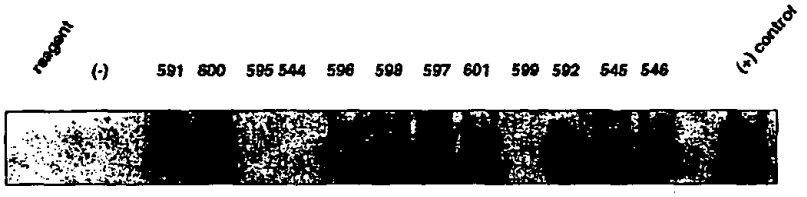


FIG. 10. Detection of *neo*^{r} sequences in bone marrow from sheep transplanted *in utero* with BMNCs from *in utero*-transduced sheep (167, 177, and 182). DNA was obtained from mononuclear cells isolated from bone marrow by Ficoll-Hypaque density centrifugation. These DNA samples were then analyzed by PCR for the presence of the *neo*^{r} sequence. The reagent control consisted of all of the constituents of the PCR reaction mixture except template DNA, and the negative control (–) DNA was isolated from the bone marrow mononuclear cells of a normal control ram. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 1%. The remainder of the samples are as marked. See text for details.

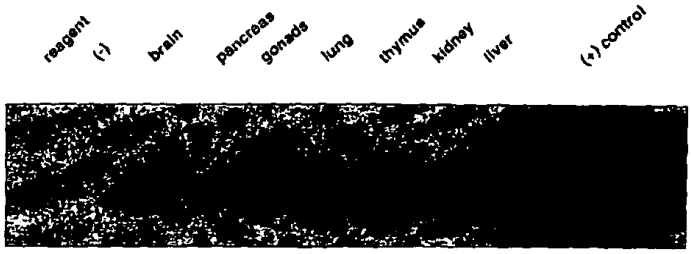


FIG. 11. Distribution of proviral DNA in tissues of animal 168 (see text for details). Organs were collected from animal 168 following sacrifice at birth. DNA was isolated from the organs as described in Materials and Methods, and was subjected to PCR with *neo*^{r}-specific primers to assess the distribution of the retroviral vector within the different tissues. The reagent control consisted of all of the constituents of the PCR reaction except the template DNA, and the negative control (–) DNA was isolated from the bone marrow mononuclear cells of a normal ram. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 10%.

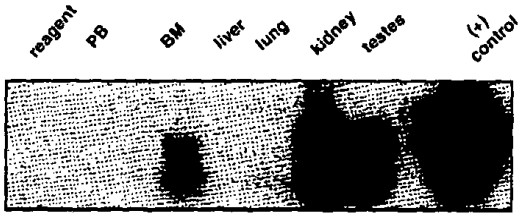


FIG. 12. Distribution of proviral DNA in tissues of animal 187 (see text for details). Animal 187 was sacrificed at 14 months posttransduction, and its organs were harvested. DNA was isolated from the organs as described in Materials and Methods, and was subjected to *neo*^{r}-specific PCR to assess the distribution of the retroviral vector within the different tissues. The reagent control consisted of all of the constituents of the PCR reaction except the template DNA. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 10%.

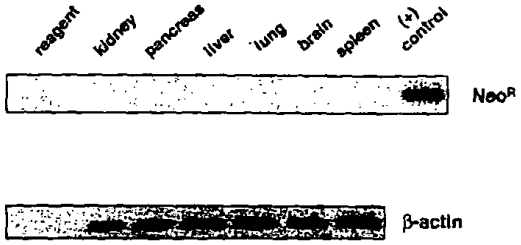


FIG. 13. Lack of transfer of the proviral DNA to the offspring of an *in utero*-transduced sheep. Ram 182 was bred with four normal control ewes, and the offspring were sacrificed and their organs analyzed for the presence of the *neo*^{r} sequence (see text for details). PCR analysis was then performed on isolated DNA from these organs. Shown here is a representative PCR from one of these offspring. The reagent control consisted of all of the constituents of the PCR reaction mixture except the template DNA. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 1%.

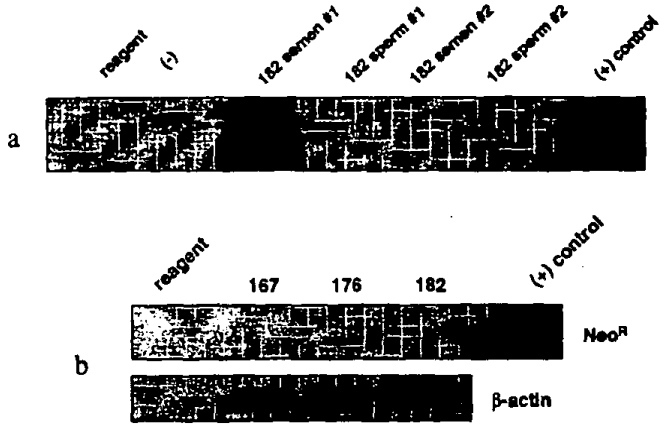


FIG. 14. (a) Absence of *neo*^{r} sequence in isolated sperm cells of animal 182. Two different ejaculates were collected from animal 182 at two different time points, and DNA was isolated and subjected to *neo*^{r}-specific PCR (labeled "semen"; see text for details). Pure populations of sperm cells were then isolated from these same two ejaculates, and these DNAs analyzed as well (labeled "sperm"). The reagent control consisted of all of the constituents of the PCR reaction mixture except the template DNA, while the negative control (–) DNA was isolated from the semen of a normal control ram. The positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 1%. (b) Absence of *neo*^{r} sequence in purified sperm cells from rams 167, 176, and 182 at 3.5 years after *in utero* transduction.

TABLE 2. FACS ANALYSIS OF NPT EXPRESSION IN PERIPHERAL BLOOD OF *IN UTERO* TRANSDUCED SHEEP[a]

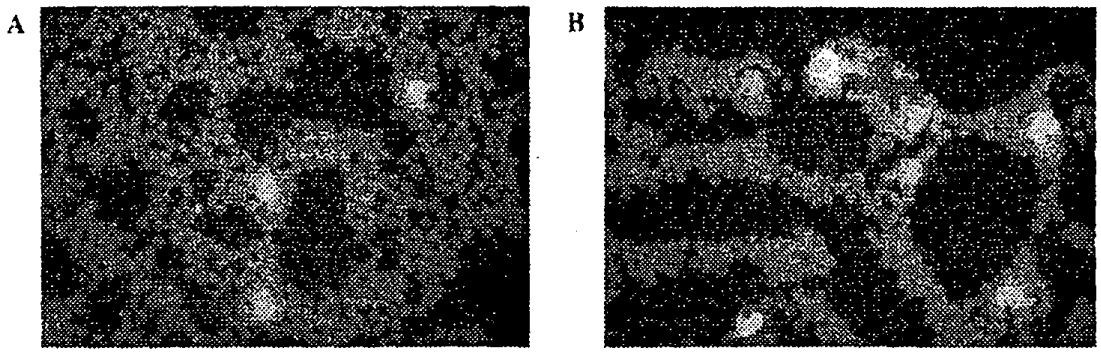
Sheep No.	*Total PB white cells*	*Lymphocytes*	*Granulocytes/ monocytes*
173	0	0.17	0
176	0	0.06	0
177	0.16	0	13.1
179	4.85	0	11.12
182	1	1.59	0
189	1.6	1.55	0.6

[a]Data obtained from FACS analysis of peripheral blood white cells from *in utero*-transduced sheep, using an antibody to NPT. Values represent the percentage of positive cells in each gated cell population after subtracting the values obtained, using the same gates, from a control sheep.

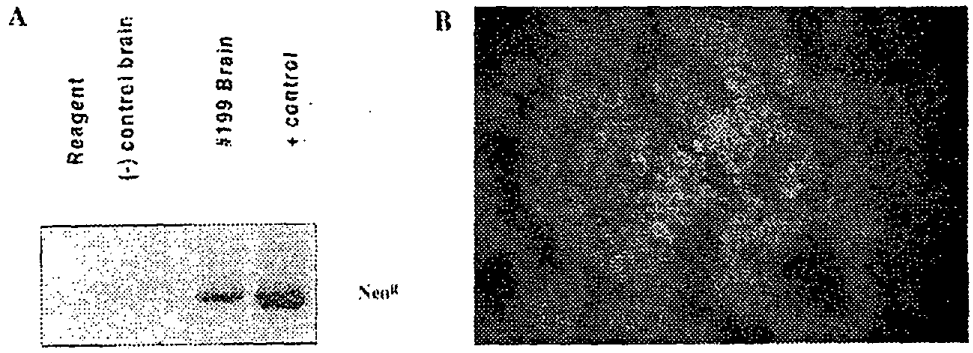
TABLE 3. CLONOGENIC ASSAY OF SECONDARY RECIPIENTS[a]

	Percentage of colonies G418 resistant		
Animal No.	*CFU-GM*	*CFU-E*	*BFU-E*
595	6.5 ± 1.9	2.6 ± 1.2	5.1 ± 2.6
596	2.5 ± 1.4	1.4 ± 0.7	7.2 ± 4.4
597	16.6 ± 1.6	4.8 ± 1.7	12.3 ± 6.0
598	4.3 ± 1.0	9.1 ± 6.0	8.6 ± 5.8
599	0	4.6 ± 0.9	23.4 ± 7.9

[a]Bone marrow mononuclear cells were obtained at 1 year posttransplant from sheep transplanted *in utero* with BMNCs from sheep 167, and clonogenic assays were performed in the presence of lethal concentrations of G418. Results are presented as the percentage of G418-resistant colonies of each lineage, after subtraction of the minimal resistance seen with BMNCs obtained from a normal age-matched control sheep.



COLOR PLATE 1. NPT activity detected by immunofluorescence of peripheral blood smears. Shown in (A) is a representative peripheral blood smear from a normal control sheep; (B) a representative smear from animal 1S2. The antibody for NPT is labeled in green with FITC, while the red counterstain is propidium iodide.



COLOR PLATE 2. Detection of *neo*^{r} sequence in brain tissue of an *in utero*-transduced lamb. (A) Animal 199 was sacrificed at 20 months posttransduction and its organs collected. The brain was rinsed extensively with PBS to remove blood, and DNA was then isolated. This DNA was then analyzed by PCR with primers specific for the *neo*^{r} sequence. The negative control DNA was isolated from the brain of a normal control animal, and the positive control consisted of the plasmid pUC18Neo diluted in normal sheep DNA to 1%. (B) After sacrifice and removal of organs, the brain of animal 199 was washed extensively with PBS and fixed for 2 days in a 10% buffered formalin solution, at which time it was paraffin-embedded, sectioned, and fluorescence *in situ* hybridization (FISH) with a *neo*^{r}-specific probe was performed. As can be seen, a strong fluorescent signal was detected in the brain. Using this same technique, we were also able to demonstrate the presence of the provirus within the liver of animal 199 (data not shown).

Human Gene Therapy 7:1123-1129, June 1996

Molecular Analysis of T Lymphocyte-Directed Gene Therapy for Adenosine Deaminase Deficiency: Long-Term Expression *In Vivo* of Genes Introduced with a Retroviral Vector

CRAIG A. MULLEN,(1) KAREN SNITZER,(2) KENNETHW.CULVER,(2) RICHARD A. MORGAN,(2) W. FRENCH ANDERSON,(3) and R. MICHAEL BLAESE(2)

ABSTRACT

Peripheral blood lymphocytes from a patient with adenosine deaminase (ADA) deficiency were transduced *in vitro* with a replication-defective retroviral vector containing a human ADA-cDNA. Eighteen months after the last of a series of infusions of autologous retroviral vector-treated cells, vector sequences were detectable in DNA isolated from peripheral blood mononuclear cells (PBMCs), with an average copy number approaching one per cell. Increased ADA enzyme activity reaching approximately one-quarter normal levels was found in this population of cells. Other evidence of long-term retroviral vector expression *in vivo* included neomycin phosphotransferase (NPT) activity and demonstration of persistent vector mRNA by reverse transcriptase polymerase chain reaction (RT-PCR). No evidence of spontaneous reversion of either mutant endogenous ADA allele was found.

OVERVIEW SUMMARY

Children with severe combined immunodeficiency due to adenosine deaminase (ADA) deficiency have been treated with autologous T lymphocytes transduced *in vitro* with a retroviral vector containing both a normal human cDNA for ADA and a cDNA for neomycin phosprotransferase (neo). This report by Mullen *et al.* analyzes long-term expression of the retroviral vector genes in the first patient over a three and one-half year period of treatment and observation.

INTRODUCTION

ISOLATION AND CHARACTERIZATION OF GENES responsible for inherited diseases and development of gene transfer technology have led to the initiation of clinical trials of gene therapy for human disease. A prerequisite for successful gene therapy of many human diseases is stable, long-term expression *in vivo* of an exogenous gene in a physiologically relevant quantity. Critical to such an outcome are factors such as the lifespan of the transduced target cell *in vivo* and the continued expression of the introduced gene *in vivo*.

Adenosine deaminase (ADA) deficiency is an autosomal recessive disorder that accounts for roughly 20% of the cases of severe combined immunodeficiency (Hirschhorn, 1990). Lack of ADA enzyme activity leads to the accumulation of adenosine, deoxyadenosine, and their metabolites, which are preferentially toxic to lymphocytes. The ADA gene was cloned in the early 1980s (Daddona *et al.*, 1984; Valerio *et al.*, 1985; Wiginton *et al.*, 1984), and preclinical gene transfer studies using this gene have been carried out by a-number of groups (Williams *et al.*, 1986; Kantoff *et al.*, 1987; Palmer *et al.*, 1987; Cournoyer *et al.*, 1991; van Beusechem *et al.*, 1992; Moritz *et al.*, 1993). One approach to treatment of this disorder is *ex vivo* retroviral vector transfer of a functional ADA gene into T lymphocytes, expansion of these cells *in vitro*, and reinfusion of these autologous genetically modified cells (Kantoff *et al.*, 1986; Culver *et al.*, 1990; Ferrari *et al.*, 1991; Braakman *et al.*, 1992). Two ADA-deficient patients were treated in this manner. Patient 1 exhibited significant lymphocyte transduction and an unequivocal increase in ADA activity. Patient 2 had less than 1% vector transduced lymphocytes and no

(1)Department of Experimental Pediatrics, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.
(2)Clinical Gene Therapy Branch, National Center for Human Genome Research, National Institutes of Health, Bethesda, MD 20892.
(3)Gene Therapy Laboratories, Norris Cancer Center, University of Southern California School of Medicine, Los Angeles, CA 90033.

significant increase in ADA activity (Blaese *et* al., 1995). Despite this, they exhibited similar increases in immunological function. Their clinical courses and the relative contributions of retroviral ADA gene transduction and infusion of activated lymphocytes expanded *in vitro* are described elsewhere (Blaese *et al.*, 1995). This report describes the long-term expression of an ADA gene in Patient 1 who received 11 such infusions over 23 months and who was followed without further intervention for an additional year and a half (Blaese *et al.*, 1990). We wished to learn how long lymphocytes transduced with retroviral vectors would persist *in vivo* and how well the genes encoded by such a vector would be expressed over time *in vivo.* Furthermore, we sought to demonstrate that the ADA found in the patient's T cells represented an authentic vector-derived gene product rather than enzyme production from an endogenous ADA gene (Brown *et al.*, 1994).

MATERIALS AND METHODS

Transduction of patient lymphocytes

The clinical protocol is described in detail elsewhere (Blaese *et al.*, 1990). Informed consent was obtained from the parents of each patient on the protocol. Each treatment consisted of infusion of approximately 1-2 *X* 10(10) autologous lymphocytes that had been exposed to LASN retroviral vector *in vitro* and cultured for a total of 10-11 days. Transduction efficiencies were usually in the range of 1-10% (Blaese *et al.*, 1995). The LASN vector is a replication-incompetent retroviral vector whose general structure is: [MoMLV LTR-human ADA cDNA-SV40 early region promoter-neomycin phosphotransferase gene] (Hock *et al.*, 1989). Clinical-grade vector was produced in PA317 amphotropic packaging cells (Miller *et al.*, 1986) (Genetic Therapy, Inc., Gaithersburg, MD).

ADA assay

ADA enzyme activity assay was performed as previously described (Kohn *et al.*, 1989). Peripheral blood mononuclear cells (PBMCs) were purified on a Ficoll gradient and washed in phosphate-buffered saline (PBS). Tissue culture cells were harvested and washed in PBS. Cells were centrifuged at ~ 1,500 *X* g in microfuge tubes and these pellets were stored at –70 C until assayed. Cells were resuspended at a concentration of 5 *X* 10(6) cells/ml in 100 mM Tris pH 7.4 and 1 mg/ml bovine serum albumin (BSA) and lysed by five rapid freeze-thaw cycles. Lysates were microfuged at ~ 15,000 X g for 2 min. A 10-ul amount of sample lysate containing the equivalent of 50,000 cells was incubated with 10 ul of 0.167 mM [(14)C]adenosine (50 uCi/ml)(Sigma, St. Louis, MO) for 15 min. (The final concentration of adenosine in the reaction was 0.083 mM.) The enzyme reaction was terminated by addition of 20 ul of absolute ethanol and heating to 95 C for 5 min. Next, 50% of the sample was applied to a cellulose TLC sheet along with 3 ul of a nonradioactive solution of 10 mM deoxyadenosine, hypoxanthine, and deoxyinosine markers (Sigma, St. Louis, MO). The developing buffer was an aqueous solution with Na(2)PHO(4)0.06 M pH 6.8, n-propyl alcohol 1.2%, and saturated ammonium sulfate 26% (vol/vol). After development and drying, the deoxyadenosine, hypoxanthine, and deoxyinosine spots were identified under shortwave UV light and cut from the sheet. Hypoxanthine and deoxyinosine (the deoxyadenosine metabolic products) were counted together while deoxyadenosine was counted separately in a liquid scintillation counter. Activity is expressed as nanomoles of adenosine deaminated/min per 10(8) cells. Duplicate samples were run in the presence of the ADA enzyme inhibitor EHNA (30 uM) (gift of R. Agbaria and D.G. Johns, Laboratory of Medicinal Chemistry, National Cancer Institute). Specific ADA activity was calculated as total adenosine deaminating activity minus EHNA-resistant activity. EHNA-resistant activity represents metabolic activity of a

nonspecific aminohydrolase present in human cells (Schrader *et al.*, 1978; Daddona *et al.*, 1981; Ratech and Hirschhorn, 1981). Positive control cells were obtained from healthy normal donors and yielded on average 72 units (normal range 66-102 units).

Neomycin phosphotransferase (NPT) assay

This was performed as previously described (Reiss *et al.*, 1984) and represents ability of lysates of 5 *X* 10(6) cells to phosphorylate a neomycin analog *in vitro*. The strongest signal in patient samples was similar to the signal generated by a 1:100 to 1:1,000 dilution of K562-LASN cells, a strong positive control. The limit of detection is approximately 1 ng of purified NPT enzyme.

Detection of vector DNA

Southern Blots: A total of 10 ug of DNA was digested with Sst I and hybridized with a 728-bp *Nco* I fragment from LASN corresponding to the SV40 promoter and *neo* gene. DNA from K562-LASN cells served as positive control.

PCR fro ADA Vector: A 10-ul amount of cell lysate (the equivalent of 5 X 10(4) cells) was used as template for a 30-cycle PCR reaction. The oligonucleotides 5'-CAGCCTCTGCAGGGCAGAAC-3' (corresponding to the 3' end of the ADA gene in LASN) and 5'-GCCCAGTCATAGCCGAATAG-3' (complementary to 5' end of the neomycin phosphotransferase gene in LASN) were used as primers. 32p-labeled dCTP was present in the reaction. Products were run on a 6% polyacrylamide gel that was dried and used to expose film.

RT-PCR analysis for LASN vector transcripts

A 3-ug amount of poly(A)RNA was treated with DNase and reverse transcribed. Then 0.3 ug of cDNA was amplified with LASN vector-specific primers (5'-CAGCCTCTGCAGGGCAGAAC-3' corresponding to the 3' end of the ADA gene in LASN and 5'-GCCCAGTCATAGCCGAATAG-3' complementary to the 5' end of the neomycin phosphotransferase gene in LASN. Following electrophoresis and blotting, the sequences were hybridized with a 527-bp probe corresponding to the entire length of the predicted PCR product.

RT-PCR analysis of endogenous (nonvector) ADA-mRNA

A 0.3-ug amount of cDNA prepared from DNase-treated poly(A)RNA was amplified with primers corresponding to exon 5 of normal ADA (found in both endogenous and vector ADA)

and antisense to a distal untranslated portion of exon 12 (found only in endogenous ADA). The upstream primer was from exon 5 (~ bp 376 relative to the start codon) and consisted of the sequence: 5'-CCAGACGAGGTGGTGGC-3'. The downstream primer was 5'-GACTATTGAGATCATGGTCTTCTT-3' and corresponded to a normally untranslated region of exon 12 (~ bp 1,175) not present in the vector cDNA. Each PCR product was split into three aliquots and digested with no enzyme (--), *Bst XI* (X), or *Bgl II* (G). Following electrophoresis and blotting of the digest products, the filter was hybridized with a 345-bp *Bam HI-Bg III* fragment from LASN corresponding to exons 9-12 of ADA.

Cloning of lymphocytes

Clones were derived from PBMCs on day 985, 9 months after the final treatment. They were isolated by limiting dilution (Nutman, 1991) and expanded in tissue culture. Peripheral blood was fractionated on a gradient of Ficoll-Hypaque. PBMCs were activated with OKT3 (10 ng/ml) and interleukin-2 (IL-2) (100 IU/ml) and initially grown on 1.5 X 10(4) 2,500 cGy irradiated allogeneic PBMCs at 0.1-10 patient cells per well in RPMI with 10% FCS in round-bottomed 96-well plates. Colonies emerging from plates with fewer than 32 positive wells were considered clonal. Culture time averaged 2 months and approximately 12 X 10(6) cells for each clone were generated.

Cell lines

TJF-2 is a human ADA-deficient T cell line immortalized with human T lymphotrophic virus type 1 (HTLV-1) (Kohn *et al.*, 1989). K562 is a human erythroleukemia cell line with ADA activity similar to that of normal human PBMCs (Table 1). These cell lines were transduced *in vitro* with LASN retroviral vector and selected in G418 1 mg/ml. The cultured pretreatment patient lymphocyte line represented Ficoll-Hypaque-purified venipuncture blood obtained before the first infusion of vector-treated cells. These PBMCs were stimulated with PHA, grown 4 weeks *in vitro*, and cryopreserved for 4 years.

RESULTS

Transduction and sampling of lymphocytes

Peripheral T lymphocytes from a previously described patient with ADA deficiency (Hershfield *et al.*, 1993) were transduced with the LASN retroviral vector containing a human ADA cDNA (Hock *et al.*, 1989). *In vitro* transduction efficiencies were 1-10% as assessed by PCR on cell product samples obtained prior to infusion

TABLE 1. ADA ENZYME ACTIVITY IN UNCULTURED PBMCs AND IN LONG-TERM LYMPHOCYTE CELL LINES

Cells

Vector DNA(a)	*Vector RNA(b)*	*Percent normal ADA activity(c)*
Uncultured PBMCs		
Cell lines		
Normal donor		

Patient pretreatment	+	NT d	100
Patient day 115	+	NT	1
Patient day 500			3
Patient day 1252			11
Patient clone 1.1			18
Patient clone 1.3			61
Patient clone 1.7			60
Patient clone 1.14			141
Patient clone 1.20			74
Patient clone 1.21			43
Cultured patient			24
Pretreatment			10
TJF-2			1
TJF-2-LASN			407
K562			98
K562-LASN			3,387

Uncultured PBMCs were obtained from Ficoll-Hypaque-treated venipuncture blood. Patient clones were obtained from patient on day 985, 9 months after the final infusion of treated cells. Cultured patient pretreatment cells were PBMCs obtained prior to the first treatment that were stimulated with PHA *in vitro* and grown *in vitro* for greater than 7 weeks in tissue culture before analysis.

(a)Vector DNA +, Cells were positive for LASN vector DNA by Southern hybridization analysis of genomic DNA and by PCR using primers specific for vector.

(b)Vector RNA +, DNase-treated mRNA was RT-PCR positive for LASN vector using primers that were specific for LASN and that did not amplify cellular ADA mRNA.

(c)% normal ADA activity, EHNA-sensitive adenosine deaminating activity of cells as a percentage of normal peripheral blood PBMC activity. % normal ADA activity values for clones represent total adenosine deaminating

activity; the limited number of cloned cells precluded repetition of the assay with the use of EHNA as a specific inhibitor of ADA.

(d)NT, Not tested due to lack of a sufficient number of cloned cells for RNA preparation.

(Blaese *et al.*, 1995). Three infusions (#3 on protocol day 60, #8 on day 314, and #11 on day 707), each followed by a significant interval before subsequent infusions resumed, were studied in detail with respect to the presence of vector DNA, vector message measurable by RTPCR, and vector product measured as ADA and neomycin phosphotransferase-2 (NPT) enzyme activity. For each of these cycles, PBMCs were sampled immediately prior to and at various times after cell infusions. These treatment-free evaluation periods were 55, 186, and 545 days, respectively.

Detection of retroviral vector DNA in patient lymphocytes

Vector sequences were readily detectable on Southern blots of this patient's PBMCs up to 545 days after the previous treatment. Both Southern blots (Fig. 1) and, semiquantitative PCR (data not shown) indicted that the average copy number of vector sequences per cell increased progressively during the course of the treatment protocol and approached approximately one in the samples taken after the eleventh infusion cycle. The vector signal did not wane during the 186 days following infusion 8, protocol days 314-500, or in the 545 days fol-

Southern

Infusion 3: NPT

RT-PCR

Southern

Infusion 8- NPT

RT-PCR

Southern

Infusion 11: NPT

lowing infusion 11, protocol days 707-1,252. Southern blots in which patient PBMC DNA was digested with *Eco* RI (which cuts only once in LASN) gave no evidence of a monoclonal or oligoclonal population of vector-containing cells (Blaese *et al.*, 1995).

ADA activity

ADA enzyme activity also increased progressively in the population of post-treatment PMBCs, approaching one-quarter normal levels in the period following the final infusion of ADA vector-modified cells (Fig. 1). This level of ADA activity is clinically significant. The increase in ADA enzyme activity in the post treatment PBMC samples suggested that the transduced LASN vector was its source. However, the ADA enzyme assay cannot distinguish between enzyme produced by the retroviral vector and endogenous ADA genes. Expression from the LASN vector was further tested in two ways: first, by measurement of NPT activity in the PBMCs and second, by detection of vector mRNA.

Expression of vector neo *gene*

The LASN vector contains two transgenes: human ADA driven by the promoter/enhancer in the MoMLV retroviral LTR and a neomycin-resistance (*neo*) gene expressed by an internal SV40 early region promoter.

.1-1 1 4~

ADA 1

-11 days
(D49)

4
D49.60 D49>60
Cuftured . LASN

4%*'*

2

55 days 1 copy0.5 copy
(Di 15) DNA-STD DNA-STD

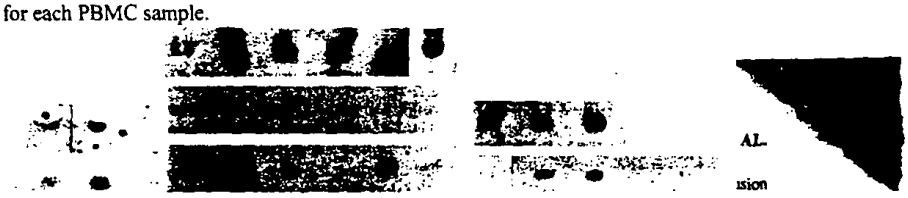
ADA 10 34 9 4 14 8
-10dayS 03D4>314 32 Clays 67 days 102 days 186 days
(D304) . LASN (D346) (0381)(D416) (D500)

61'~,4L #b , 'e*

RT-PCR Aw

ADA 8 1114 17 13
-11 days D696,707 D696~707 24 days 109days Wdays
(D696) cuftured + LASN (D731) (D816) (012521

FIG. 1. LASN vector expression in patient PBMCs. Patient PBMC samples from infusion cycles 3, 8, and 11 were studied. The study date is indicated under each column. The number of days from the initiation of the study is indicated by "D" *(e.g.,* D115). The number of days relative to the infusion date for the infusion cycle is also indicated *(e.g.,* "55 days" relative to infusion 3). PBMCs from blood were directly assayed and are identified with a "D" number in parentheses, *e.g., (D115).* Cells grown in culture without additional vector are designated "cultured." Those designated "LASN" were exposed to additional vector and were aliquots of the cells infused into the patient in the infusion cycle. For each point in time, a Southern blot was performed to identify the LASN vector. DNA from K562-LASN cells provided the positive control. NPT is neomycin phosphotransferase activity in lysates of 5 X 10(6) cells. Positive patient samples are similar in intensity to samples of 5 X 10(3) K562-LASN cells. RT-PCR represents LASN vector message in DNase-treated RNA. Positive patient signals from 0.3 ug of cDNA were similar in intensity to 0.3 ng of cDNA from K562-LASN. EHNA-sensitive ADA activity is described for each PBMC sample.



neo gene activity is not normally present in human cells and thus the presence of its product, NPT, in cells would demonstrate transcription and translation of integrated vector sequences. PBMCs from the same days that were analyzed for vector presence by Southern blots were assayed for NPT activity (Fig. 1). NPT could be directly detected in samples from the patient's circulating T cells on several occasions during the course of our observations, confirming vector derived gene function.

Expression of vector ADA gene

Demonstration of NPT enzyme activity in the patient's T cells provides unambiguous evidence of vector expression. However, the *neo* and ADA genes are in separate transcriptional units and it could be argued that the ADA activity still could come from the patient's endogenous ADA genes. Conventional Northern blots were insensitive to the levels of vector ADA-mRNA in the PBMCs, compatible with the relatively modest amount of ADA and NPT activity in these cells. RT-PCR, however, detected vector ADA message in all the post-treatment PBMC samples with the exception of days 115 and 381 (Fig. 1).

Comparison of LASN vector expression in patient lymphocytes and in transformed cell lines

Increased ADA activity, NPT activity, and vector mRNA were found in patient PBMCs after treatment and the relative levels of these correlated with each other. In these studies, transformed human cell lines K562 and TJF-2 transduced with the LASN vector and selected in G418 were used as positive controls. In these cell lines, the vector-induced increases in ADA activity (Table 1) were much greater than in the patient's lymphocytes. Significantly greater NPT activity and vector mRNA were also seen in these cell lines (data not shown).

Analysis of endogenous ADA activity

As part of this post-treatment analysis, an attempt was made to clone patient lymphocytes and analyze the magnitude of vector expression in clonal populations. T cell clones were prepared by limiting dilution, without G418 selection, from the patient's peripheral blood drawn on protocol day 985. The patient's cells did not clone efficiently, but six clones yielded enough material before reaching senescence for characterization by Southern, RT-PCR, and ADA enzyme analyses. A summary of the analysis is contained in Table 1. Five of the six clones contained integrated LASN vector DNA. Each of the vector-positive clones exhibited significant ADA enzyme activity, ranging from 43% to 141% of that in normal PBMC (Table 1). Vector mRNA was detected in each of the three vector-positive clones analyzed. Clone 1.21 was negative for vector DNA by both Southern blot and PCR, and also negative for vector mRNA by RT-PCR. Surprisingly, clone 1.21 had 24% normal adenosine deaminating activity.

It is possible that the process of cloning may have selected for cells that had higher levels of adenosine deaminating activity. Consistent with this was the finding that a cell line derived from nontransduced, pretreatment PBMCs from this patient also contained some adenosine deaminating activity (Table 1). This cell line had been stimulated with phytohemoagglutinin (PHA) and IL-2, grown for 4 weeks, cryopreserved for 4 years, thawed, and grown again for 3 weeks. PBMCs obtained by venipuncture from the patient prior to therapy and not cultured had never shown any significant ADA activity. We and others have observed that upon occasion T cells from ADA(-)SCID patients will exhibit increased ADA enzyme activity upon *in vitro* activation and culture (Arredondo-Vega *et al.*, 1990). This finding raised the possibility that the increased ADA activity in post-treatment patient PBMCs might represent recovery of expression of an endogenous ADA gene or amplification of another enzyme that could deaminate adenosine.

In normal cells adenosine deaminating activity is largely due to ADA enzyme, whereas a nonspecific aminohydrolase contributes approximately 1% of the activity. These two enzymes can be distinguished by use of the enzyme inhibitor EHNA (Schrader *et al.*, 1978; Daddona *et al.*, 1981; Ratech and Hirschhorn, 1981). ADA is inhibited by EHNA whereas the nonspecific aminohydrolase is not. (All ADA values in Fig. 1 are EHNA sensitive.) In theory cells overexpressing either enzyme would have a survival advantage as they were activated and expanded *in vitro* for 10 days as part of the treatment protocol. To investigate this possibility, cultured pretreatment cells that had shown ADA activity were reassayed with the use of EHNA as a specific inhibitor of ADA enzyme (Table 1). It was found that half of the adenosine deaminating activity in these cultured cells was EHNA resistant and therefore represented amplified activity of the nonspecific aminohydrolase. Nonetheless, some authentic, EHNA sensitive ADA enzyme activity was also present in these cultured cells.

This raised the possibility that in the course of treatment a spontaneous reversion of the mutations in the patient's cells may have occurred. The mutations for this patient have been described (Santisteban *et al.*, 1993). One mutant allele (A) contains a Gly216 > Arg point mutation in exon 7 that creates a new *Bst* XI restriction site. A patient homozygous for this point mutation had less than 1% normal ADA activity and a severe clinical immunodeficiency (Hirschhorn *et al.*, 1991). The other allele (B) is a splice site mutation in intron 5 (a T + 6 > A transversion) that eliminates exon 5 from mRNA, resulting in a mRNA reading frame shift and a premature stop signal at codon 131 (in the middle portion of the protein).

Given the nature of the splice site mutation, it could be speculated that correct splicing might occur in culture-activated lymphocytes and be responsible for producing normal ADA protein. To evaluate this possibility, PBMC samples from five time points were subjected to RT-PCR using primers from exon 5 and a distal untranslated portion of exon 12. This primer set will not amplify vector sequences because the vector lacks this untranslated portion of ADA exon 12 and will not amplify improperly spliced mRNA from the endogenous allele B because such splicing eliminates exon 5. If proper splicing were occurring, two species of RT-PCR products should be seen in the PBMCs: one possessing the extra *Bst* XI site (from allele A with the point mutation) and one lacking this site (allele B). Following *Bst* XI digestion, mutant A yields a 485-bp product whereas the wild-type allele would yield a 596-bp product. Figure 2 demonstrates that in none of the samples analyzed was there evidence of properly spliced mRNA lacking the point mutation as judged by the lack of the 596-bp *Bst* XI-digested RT-PCR product. Thus, it is very unlikely that the increased ADA activity *in vivo* came from

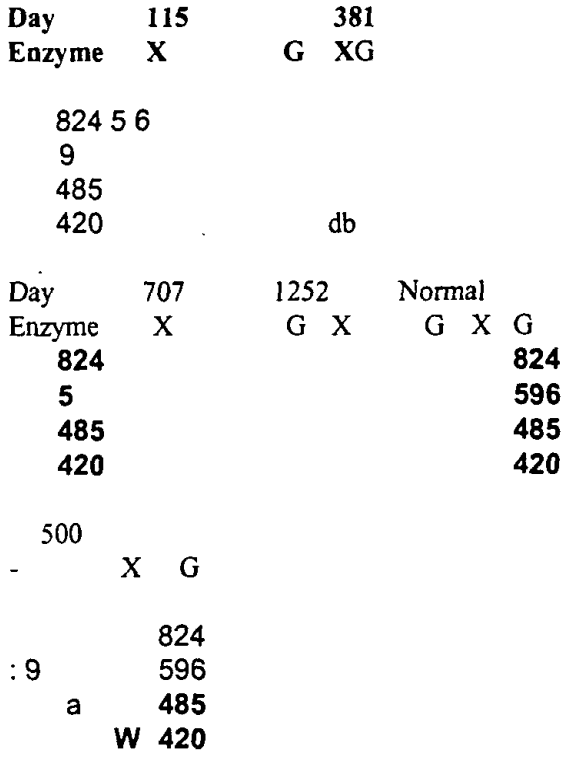


FIG. 2, RT-PCR analysis of endogenous (nonvector) ADAmRNA. A 0.3-ug amount of cDNA was amplified with PCR using vectors that were specific for endogenous ADA and that fail to amplify LASN vector-derived mRNA. Each PCR product was split into three aliquots and digested with: no enzyme *(-), Bst* XI (X) or *Bgl* II (G). Normal and mutant ADA genes should both produce an 824-bp band that reduces to 420 bp with *Bgl* II digestion. (The larger bands in the "G" lanes represent incomplete digestion with *Bgl* II.*) Bst* XI digestion of the PCR product from a normal gene yields a 596-bp band, whereas the mutant ADA gene which contains an additional *Bst* XI site yields a 485-bp band. Samples are from the patient on the study day indicated or from a normal human donor. The bands corresponding to days 115, 381, and 500 represent an overnight exposure of the blot whereas the bands from days 707, 1,252, and the normal donor are from a 3-hr exposure of the same blot.

reversion of either the point mutation in the one gene or proper splicing of the allele with the splice site mutation. The analysis does not exclude the possibility that some post-translational modification of the Gly216 > Arg mutant enzyme contributes to the increased ADA activity in the vector-negative cultured T cells, but no precedent for such a mechanism has been reported.

DISCUSSION

This molecular analysis of the extent and duration of expression of transgenes introduced into human peripheral T cells with retroviral vectors has demonstrated that prolonged transgene expression *in vivo* is possible with the current generation of retroviral vectors when used in primary T cells. It has been observed in some experimental systems that in nontransformed cells retroviral vector genes expressed *in vitro* may cease to be expressed when the vector-modified cells are returned to the living host (Palmer *et al.,* 1991). This

experimental observation, as well as the observation that some vector-negative lymphocyte lines from this ADA-deficient patient exhibited some ADA activity, made it important to evaluate expression of the retroviral ADA vector critically.

The demonstration of NPT activity and vector mRNA in the patient's lymphocytes provides positive evidence for long-term expression. The failure to identify any reversions from the patient's mutant ADA genes also strengthens the conclusion that the increased ADA activity in the patient's lymphocytes was due to successful transfer and expression of the vector. In these studies we observed that the magnitude of expression in patient lymphocytes was only 0.1-1% of that seen in vector-treated control K562 or TJF-2 transformed cell lines. This difference may be due to different selection pressures. The transformed cell lines were selected *in vitro* with G418 whereas the patient's lymphocytes were not. It is highly likely that *in vivo* there was selective pressure for lymphocytes with greater ADA activity. Transduction efficiencies were only 1-10%, yet over the 3 years of observation the proportion of PBMCs that contained LASN vector increased as judged by Southern blots.

An alternate explanation for differences in vector activity is that there are inherent differences between transformed and nontransformed cells that affect retroviral vector expression. If this proves to be the case, the common use of continuously growing, transformed cell lines in the design and testing of genetic vectors may be misleading if the intended use of the vectors is transduction of normal cells.

The clinical course of this and another patient treated with activated, expanded autologous lymphocytes exposed to the retroviral ADA vector has recently been described (Blaese *et al.*, 1995). The exact contribution of retroviral ADA gene transfer relative to other components of therapy *(e.g.,* infusion of activated autologous lymphocytes and ongoing treatment with parenteral ADA enzyme) to the observed clinical improvement is difficult to assess without a randomized prospective trial. Nonetheless, the clear demonstration of vector activity in this pilot study demonstrates that therapeutic retroviral vector-mediated gene transfer in humans is technically feasible and justifies other clinical trials. The observation of long-term expression of vector genes in T lymphocytes *in vivo* suggests other applications. T lymphocytes survive for many years in immunologically normal hosts. Given their longevity and the ease with which they can be collected and manipulated *in vitro*, lymphocytes may be attractive vehicles for disorders other than immunodeficiencies in which long-term gene expression and systemic protein delivery is necessary.

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Address reprint requests to:
Dr. Craig Mullen
Department Experimental Pediatrics, Box 88
U.T.M.D. Anderson Cancer Center
1515 Holcombe Blvd.
Houston, 7X 77030

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TABLE 1. ADA ENZYME ACTIVITY IN UNCULTURED PBMCS AND IN LONG-TERM LYMPHOCYTE CELL LINES

Cells		*Vector DNA*[a]	*Vector RNA*[b]	*Percent normal ADA activity*[c]
Uncultured PBMCs	Normal donor	−	−	100
	Patient pretreatment	−	−	1
	Patient day 115	+	−	3
	Patient day 500	+	+	11
	Patient day 1252	+	+	18
Cell lines	Patient clone 1.1	+	NT[d]	61
	Patient clone 1.3	+	+	60
	Patient clone 1.7	+	+	141
	Patient clone 1.14	+	+	74
	Patient clone 1.20	+	NT	43
	Patient clone 1.21	−	−	24
	Cultured patient Pretreatment	−	−	10
	TJF-2	−	−	1
	TJF-2-LASN	+	+	407
	K562	−	−	98
	K562-LASN	+	+	3,387

Uncultured PBMCs were obtained from Ficoll-Hypaque-treated venipuncture blood. Patient clones were obtained from patient on day 985, 9 months after the final infusion of treated cells. Cultured patient pretreatment cells were PBMCs obtained prior to the first treatment that were stimulated with PHA *in vitro* and grown *in vitro* for greater than 7 weeks in tissue culture before analysis.

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[b]Vector RNA +, DNase-treated mRNA was RT-PCR positive for LASN vector using primers that were specific for LASN and that did not amplify cellular ADA mRNA.

[c]% normal ADA activity, EHNA-sensitive adenosine deaminating activity of cells as a percentage of normal peripheral blood PBMC activity. % normal ADA activity values for clones represent total adenosine deaminating activity; the limited number of cloned cells precluded repetition of the assay with the use of EHNA as a specific inhibitor of ADA.

[d]NT, Not tested due to lack of a sufficient number of cloned cells for RNA preparation.

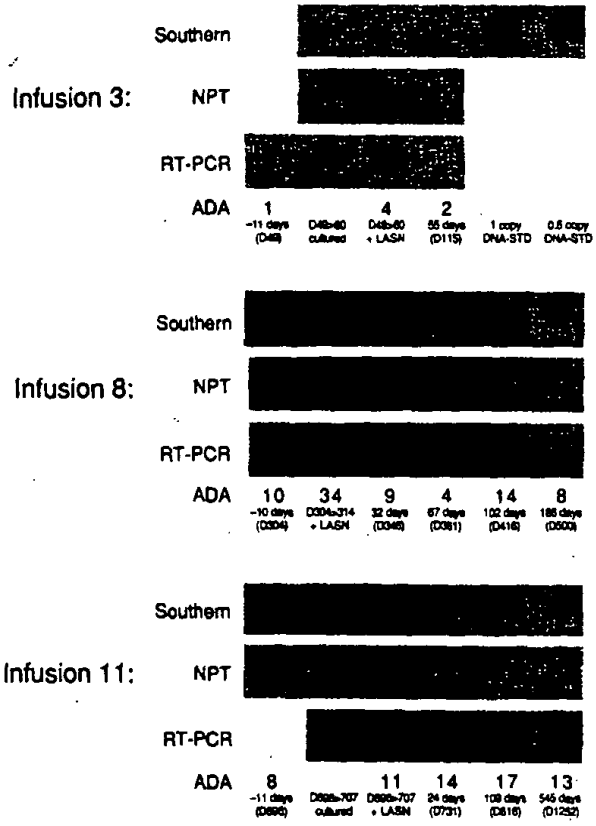

FIG. 1. *LASN* vector expression in patient PBMCs. Patient PBMC samples from infusion cycles 3, 8, and 11 were studied. The study date is indicated under each column. The number of days from the initiation of the study is indicated by "D" (*e.g.*, D115). The number of days relative to the infusion date for the infusion cycle is also indicated (*e.g.*, "55 days" relative to infusion 3). PBMCs from blood were directly assayed and are identified with a "D" number in parentheses, *e.g.*, (D115). Cells grown in culture without additional vector are designated "cultured." Those designated "LASN" were exposed to additional vector and were aliquots of the cells infused into the patient in the infusion cycle. For each point in time, a Southern blot was performed to identify the LASN vector. DNA from K562-LASN cells provided the positive control. NPT is neomycin phosphotransferase activity in lysates of 5×10^6 cells. Positive patient samples are similar in intensity to samples of 5×10^3 K562-LASN cells. RT-PCR represents LASN vector message in DNase-treated RNA. Positive patient signals from 0.3 μg of cDNA were similar in intensity to 0.3 ng of cDNA from K562-LASN. EHNA-sensitive ADA activity is described for each PBMC sample.

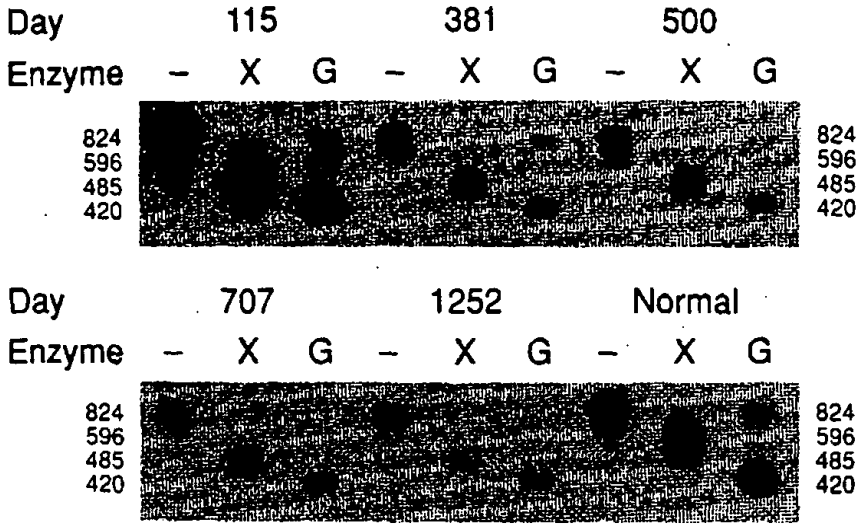

FIG. 2. RT-PCR analysis of endogenous (nonvector) ADA-mRNA. A 0.3-μg amount of cDNA was amplified with PCR using vectors that were specific for endogenous ADA and that fail to amplify LASN vector-derived mRNA. Each PCR product was split into three aliquots and digested with: no enzyme (−), *Bst* XI (X) or *Bgl* II (G). Normal and mutant ADA genes should both produce an 824-bp band that reduces to 420 bp with *Bgl* II digestion. (The larger bands in the "G" lanes represent incomplete digestion with *Bgl* II.) *Bst* XI digestion of the PCR product from a normal gene yields a 596-bp band, whereas the mutant ADA gene which contains an additional *Bst* XI site yields a 485-bp band. Samples are from the patient on the study day indicated or from a normal human donor. The bands corresponding to days 115, 381, and 500 represent an overnight exposure of the blot whereas the bands from days 707, 1,252, and the normal donor are from a 3-hr exposure of the same blot.

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Molecular Engineering of Matrix-Targeted Retroviral Vectors Incorporating a Surveillance Function Inherent in von Willebrand Factor

FREDERICK L. HALL,[1,2] LIQIONG LIU,[1,2] NIAN LING ZHU,[2] MARIA STAPFER,[2] W. FRENCH ANDERSON,[1,3] ROBERT W. BEART,[2] and ERLINDA M. GORDON[1,3]

ABSTRACT

A major obstacle that limits the potential of human gene therapy is the inefficiency of gene delivery to appropriate sites *in vivo*. Previous studies demonstrated that the physiological surveillance function performed by von Willebrand factor (vWF) could be incorporated into retroviral vectors by molecular engineering of the MuLV ecotropic envelope (Env) protein. To advance the application of vWF targeting technology beyond laboratory animals, we prepared an extensive series of Env proteins bearing modified vWF-derived matrix-binding sequences and assembled these chimeric proteins into targeted vectors that are capable of transducing human cells. Initially, a dual envelope configuration was utilized, which required coexpression of a wild-type amphotropic Env. Subsequently, streamlined "escort" Env proteins were constructed wherein the inoperative receptor-binding domain of the targeting partner was replaced by the vWF-derived collagen-binding motif. Ultimately, an optimal construct was developed that exhibited properties of both extracellular matrix (ECM)-targeting and near wild-type amphotropic infectivity, and could be arrayed as a single envelope on a retroviral particle. On intraarterial instillation, enhanced focal transduction of neointimal cells (~20%) was demonstrated in a rat model of balloon angioplasty. Moreover, transduction of tumor foci (~1–3%) was detected after portal vein infusion of a matrix-targeted vector in a nude mouse model of liver metastasis. We conclude that the unique properties of these targeted injectable retroviral vectors would be suitable for improving therapeutic gene delivery in numerous clinical applications, including vascular restenosis, laser and other surgical procedures, orthopedic injuries, wound healing, ischemia, arthritis, inflammatory disease, and metastatic cancer.

OVERVIEW SUMMARY

Toward developing targeted injectable vectors for retroviral gene therapy, we constructed a series of modified amphotropic Env proteins bearing von Willebrand factor (vWF)-derived matrix-targeting (collagen-binding) peptides, and assembled these chimeric proteins into targeted vectors that were capable of transducing human cells. Initially, a dual envelope configuration was utilized, which required coexpression of a wild-type amphotropic Env. Subsequently, streamlined "escort" Env proteins were constructed wherein the inoperative receptor-binding domain of the targeting partner was replaced by the vWF-derived collagen-binding motif. Ultimately, an optimal construct was developed that exhibited properties of both high affinity for collagen and wild-type amphotropic infectivity, and could be arrayed as a single envelope on a retroviral particle. On intraarterial instillation, enhanced focal transduction of neointimal cells *in vivo* was demonstrated in a rat model of balloon angioplasty. Moreover, transduction of tumor foci *in vivo* was observed on portal vein infusion of a matrix-targeted vector in a nude mouse model of liver metastasis. These findings represent a definitive advance in the development of targeted injectable retroviral vectors for gene therapy of vascular restenosis and metastatic cancer.

[1]Gene Therapy Laboratories, [2]Department of Colorectal Surgery, and [3]Department of Pediatrics, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90089.

INTRODUCTION

Retroviral vectors offer numerous advantages as gene delivery systems (Salmons and Gunzburg, 1993; Gordon and Anderson, 1994). Mechanistically, transduction of target cells by retroviral vectors is initiated by binding of the viral envelope protein to cell surface receptors (Hunter and Swanstrom, 1992; Weiss, 1993) followed by fusion of viral and cellular membranes (White, 1992) and internalization of the viral core. Significant advancements in the production (Soneoka *et al.*, 1995; Yang *et al.*, 1999) and physiological stability (Pensiero *et al.*, 1996) of retroviral vectors have been made. However, the potential use of these vectors for therapeutic gene delivery is severely limited by logistical considerations, principal of which is the lack of tissue specificity of current vectors and a resulting inability to deliver sufficient numbers of vector particles to target cells *in vivo* (Anderson, 1995; Verma and Somia, 1997). Thus, the development of targeted injectable retroviral vectors for human gene therapy remains a longstanding (Salmons and Gunzburg, 1993; Anderson, 1998), yet elusive (Anderson, 1995; Verma and Somia, 1997) goal.

To advance the biotechnology of retroviral vector targeting, research has focused on molecular engineering of the retroviral envelope (Env) proteins (Kasahara *et al.*, 1994; Cosset and Russell, 1996; Peng *et al.*, 1997). Our previous work on vector targeting (Hall *et al.*, 1997; Wu *et al.*, 1998) supports the general concept of strategically modifying retroviral envelopes to generate tissue- or cell-specific targeted viral vectors (Anderson, 1995; Weiss and Tailor, 1995). However, in contrast to more standard approaches to engender tissue targeting by display of polypeptide ligands (Kasahara *et al.*, 1994; Valsesia-Wittman *et al.*, 1994) or single-chain antibodies (Russell, 1993; Cosset *et al.*, 1995; Somia *et al.*, 1995) on viral envelopes, we undertook the seemingly counterintuitive strategy of targeting the extracellular matrix (ECM) itself, i.e., targeting ECM proteins exposed in pathophysiological lesions to concentrate vectors in the ECM in the vicinity of target cells (Hall *et al.*, 1997). To exploit the basic biology of wound healing, we incorporated a matrix-targeting (i.e., collagen-binding) motif derived from von Willebrand (coagulation) factor (vWF) into the ecotropic (CEE^+) murine leukemia virus (MuLV) Env to provide an integral gain-of-function (i.e., high collagen-binding affinity) without inducing conformational changes that would compromise viral infectivity (Hall *et al.*, 1997). Conceptually, molecular "tethering" of collagen-targeted viral particles to exposed ECM would enhance the effective vector concentration in the vicinity of target cells and, against formidable dilution factors, would promote binding, fusion, and core entry via natural virus–receptor mechanisms.

von Willebrand factor is a mosaic plasma glycoprotein that is synthesized by both platelets and endothelial cells and is normally found in circulating blood (Ginsburg *et al.*, 1989). Initially identified as a deficient coagulation factor in a form of inherited hemophilia (Ruggeri and Zimmerman, 1987), vWF performs a vital surveillance function by mediating platelet adhesion to sites of vascular injury. The aggregation of platelets onto the subendothelium initiates a series of biochemical reactions that ultimately results in the clotting of blood and cessation of bleeding. Discrete domains of vWF bind with high affinity to newly exposed collagen (Takagi *et al.*, 1991; Tuan *et al.*, 1996); this, together with glycoprotein Ib and glycoprotein IIb/IIIa, promotes the platelet–vessel wall interaction. The pertinent structural domains of vWF, depicting a high-affinity collagen-binding domain (CBD) within the D2 domain of the mature polypeptide, are shown in Fig. 1A. Previous studies demonstrated that transposition of vWF-derived sequences into the ecotropic (CEE^+) retroviral Env protein serves to direct retroviral recruitment and accumulation at sites of exposed collagen in an animal model of vascular injury, enhancing both the transduction of reactive smooth muscle cells (Hall *et al.*, 1997; Anderson, 1998) and the efficacy of gene therapy for vascular restenosis (unpublished observations). Realizing that this ECM-targeting technology may have important implications in the development and implementation of numerous gene therapy protocols, we generated a series of matrix-targeted amphotropic retroviral vectors for transduction of human cells.

MATERIALS AND METHODS

Cell lines and cell culture conditions

Murine NIH 3T3 and 293T cells (CRL 11268) were supplied by the American Type Culture Collection (Rockville, MD). NIH 3T3 and 293T cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (D10; BioWhittaker, Walkersville, MD). The plasmid pcgp, containing the viral *gag–pol* genes, and a retroviral vector, pcnBg, expressing a nuclear-targeted β-galactosidase construct, were kindly provided by P. Cannon and L. Li, respectively (Gene Therapy Laboratories, University of Southern California, Los Angeles, CA). The rat monoclonal antibody 83A25, directed against the C terminus of the $gp70^{env}$ protein, was provided by L. Evans (Rocky Mountain Laboratories, Hamilton, MT).

Molecular engineering and cloning of the escort series of MuLV envelope proteins bearing a collagen-binding domain

Sequence encoding the collagen-binding domain (CBD) with strategic linkers was incorporated into the CEE^+ *env* as a pair (sense and antisense) of synthetic oligonucleotides. The tandem synthetic oligonucleotides were annealed by boiling followed by gradual cooling, after which the DNA duplexes were separated from single-stranded oligonucleotides by passage through a G25 column (5 Prime → 3 Prime, Boulder, CO). The cDNA inserts were cloned into the CEE-C (Δ hinge) *env* construct, which was modified by replacement of an amphotropic proline-rich hinge region containing three unique restriction sites (*Avr*II, *Pst*I, and *Stu*I) and an additional *Ngo*MI restriction site (Wu *et al.*, 1998). The vector was cut with the following restriction enzymes to generate the respective constructs: *Bst*EII to *Avr*II (BA); *Bst*EII to *Stu*I (BS); and *Bst*EII to *Ngo*MI (BN) inserts. The linearized vectors were confirmed by restriction analysis on agarose gels and purified by the GeneClean method (Bio 101, Vista, CA) prior to ligation with the respective cDNA (CBD) inserts and T4 DNA ligase (New England BioLabs, Beverly, MA). Each construct was confirmed by restriction analysis followed by direct DNA sequence analysis.

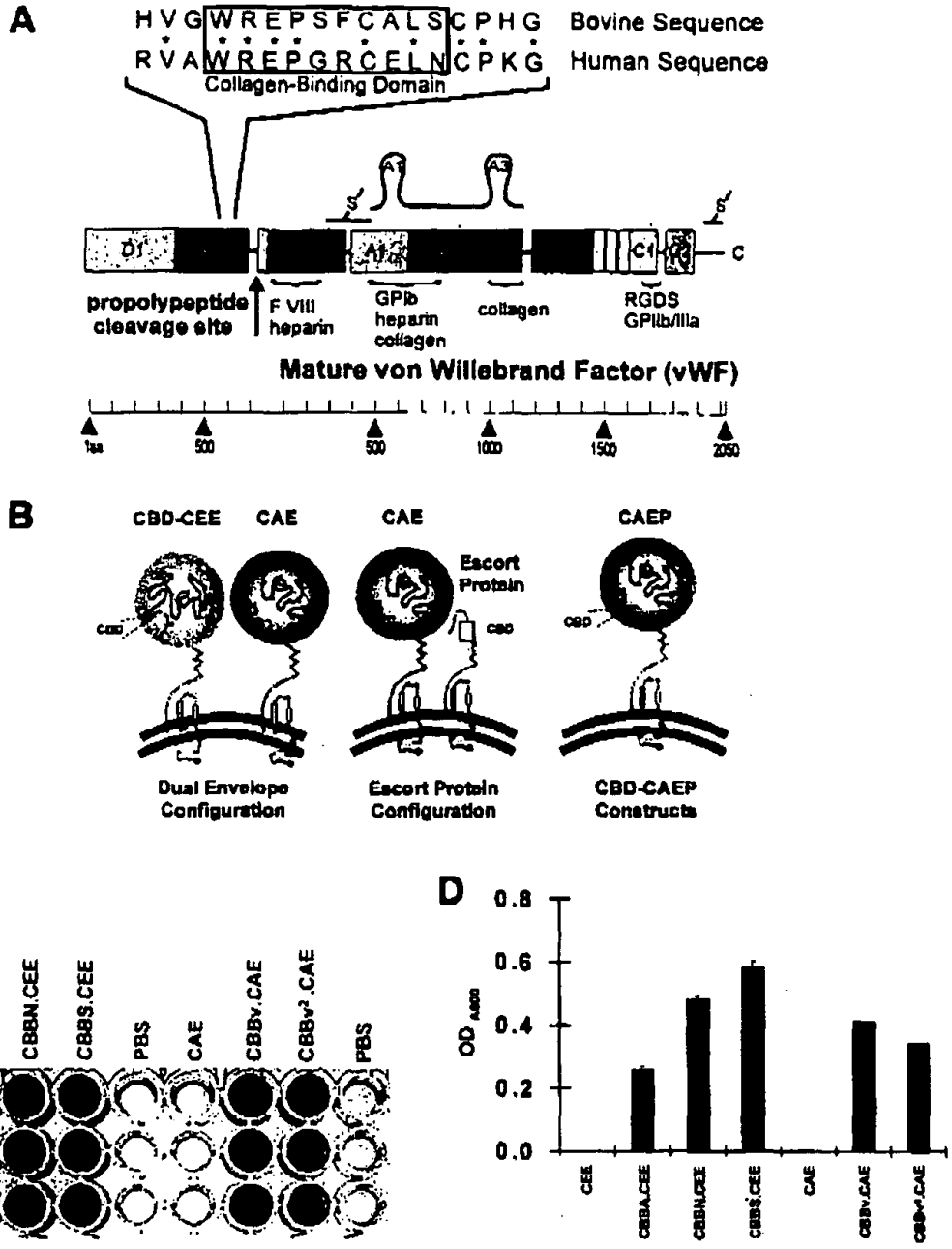

FIG. 1. (A) Structural domains of von Willebrand factor (vWF), identifying the collagen-binding motif within the D2 domain of the mature polypeptide. The mimimal collagen-binding sequences of human and bovine vWF, including the flanking residues, are shown. This figure is a modification of the mosaic protein structure of vWF, published by Montgomery *et al.* (1998). (B) Evolution of matrix-targeted amphotropic envelope designs, including dual envelope configurations, envelope "escort" proteins, and CAEP constructs, each incorporating a collagen-binding domain (CBD), as well as amphotropic trupism, into the corresponding vectors. (C and D) Binding of virions bearing chimeric CEE and CAE Env proteins onto collagen matrices. The comparative binding affinities of vectors bearing WT *env* (CEE or CAE) versus vectors displaying matrix-targeting motifs are inferred from the varying degrees to which ELISA wells are darkened (C), expressed as OD_{A650} readings on a Rainbow Spectra ELISA reader (D). CEE (wild-type CEE *env*); CBBS-CEE.CEE (collagen-binding domain at *Bst*EII and *Stu*I sites of CEE plus wild-type CEE); CBBN-CEE.CEE (collagen-binding domain at *Bst*EII and *Ngo*MI of CEE plus wild-type CEE); CBBA-CEE.CEE (collagen-binding domain at *Bst*EII and *Avr*II of CEE plus wild-type CEE); CAE (wild-type CAE *env*); CBBv-CAE.CAE (collagen-binding domain at *Bst*EII of CAE plus wild-type CAE); CBBv²-CAE.CAE (two contiguous collagen-binding domains at *Bst*EII of CAE plus wild-type CAE).

Design and engineering of the collagen-targeted amphotropic Env proteins

Figure 2 shows diagrammatically the receptor-binding domain of the 4070A Env surface (SU) protein into which the collagen-binding polypeptides were inserted into either a *Bst*EII site between amino acids 18 and 19 engineered by point mutation of the CAE *env* or a *Pst*I site (encoding Ala-Ala-Gly) added by polymerase chain reaction (PCR) between amino acids 6 and 7 of the mature CAE protein to generate CAEB (*Bst*EII) and CAEP (*Pst*I), respectively. Tandem synthetic oligonucleotides (as shown) were used to generate the respective cDNA inserts. Sequence encoding a bovine vWF collagen-binding domain, WREPSFMALS, was inserted into the *Bst*EII site (Fig. 3A), and sequence encoding bovine (Bv) or human (Hs) WREPGRMELN vWF collagen-binding domain was inserted into the *Pst*I site (Fig. 3B). The inserts were flanked by either standard linkers (LS) or linkers modified to closely approximate native vWF (LF) flanking sequences. After ligation, clone selection, and restriction analysis, the structure of each construct was verified by DNA sequence analysis.

Production of matrix-targeted retroviral vectors

High-titer vectors were generated by a transient three- or four-plasmid cotransfection system (Soneoka *et al.*, 1995), in which the packaging components *gag–pol*, the chimeric *env*, and/or the wild-type (WT) amphotropic *env* expressed from the cytomegalovirus (CMV) promoter were placed on separate plasmids, each containing a simian virus 40 (SV40) origin of replication. Simultaneous introduction of these plasmids and a retroviral vector in 293T cells expressing the SV40 T-antigen was followed by sodium butyrate treatment, and harvest of retroviral vector supernatants. The vectors coexpressing chimeric and WT Env proteins were named CB-CAE.CAE and CB2-CAE.CAE, while the vectors expressing proteins other than WT Env were named CAEP/CBBv1, CAEP/CBBv2, CAEP/CBHs1, and CAEP/CBHs2 (CB-CAE, collagen-binding domain inserted into the *Bst*EII site; CB2-CAE, two collagen-binding domains inserted into the *Bst*EII site; CAE, WT amphotropic *env*; CAEP/CB, collagen-binding domain inserted into the *Pst*I site; Bv, bovine derived; Hs, human vWF-derived; LF or 1, linkers derived from natural vWF sequences; LS or 2, standard linkers).

Viral Env protein expression and virion incorporation assays

The level of expression of the mature $gp70^{env}$ protein in cell lysates of WT CAE and chimeric eco- and amphotropic Env proteins bearing matrix-targeting (collagen-binding) motifs was evaluated by Western analysis (Zhu *et al.*, 1998). For evaluation of virion incorporation, the retroviral supernatant was purified on a 20% sucrose gradient, and the viral pellet was subjected to Western analysis with a rat monoclonal antibody, 83A25, to detect the wild-type and chimeric Env proteins, while anti-p30 antiserum directed against the retroviral Gag protein served as a control (Zhu *et al.*, 1998).

Viral titers

Viral titers were determined and quantified on the basis of expression of the β-galactosidase reporter gene (Skotzko *et al.*, 1995). Briefly, 2.5×10^4 NIH 3T3 cells were plated in each well of six-well plates 1 day prior to transduction. Medium was replaced with 1 ml of serial dilutions of viral supernatant with Polybrene (8 μg/ml) for 2 hr. One milliliter of fresh D10 was added to the cultures, which were then maintained overnight at 37°C in 5% CO_2. Medium was replaced with fresh D10 and cultures were maintained for an additional 24 hr. Expression of β-galactosidase in the respective cultures was evaluated by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining 48 hr after transduction of the NIH 3T3 cells. As expected, the infectious titers of both chimeric and WT CAE vectors ranged from 10^6 to 10^7 CFU/ml, depending on the amount of plasmid DNA used in the transfection protocol.

Collagen-binding and transduction assays

Collagen coating. In six-well cell culture plates, 500 μl of 3% collagen was applied with rocking to each well and the plate was incubated at room temperature for 2 hr in a tissue culture hood. The collagen solution was aspirated and discarded, and the plates were allowed to dry for 1 min under UV light. Each well was then washed twice with copious amounts of 1 × phosphate-buffered saline (PBS).

Collagen binding. After washing the collagen-coated wells, 1 ml of the experimental vector was then placed in each well and the wells were incubated at 37°C in 5% CO_2 for 30 min. The vector was aspirated and discarded, and the wells were washed twice with PBS, prior to blocking with bovine serum albumin (BSA) and incubation with 300 μl (hybridoma supernatant) of a rat monoclonal antibody (83A25) directed against the C terminus of the gp70 MuLV Env protein (Hall *et al.*, 1997) at room temperature for 1 hr. The wells were again washed twice with PBS, and then incubated in 500 μl of horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (diluted 1:2500; Zymed, South San Francisco, CA) at room temperature for 30 min. After washing, the wells were incubated in 500 μl of rat peroxidase–anti-peroxidase antibody (diluted 1:1000; Sternberger Monoclonals, Lutherville, MD) at room temperature for 30 min and the intensity of the color reaction (blue) was read at OD_{650} on a Rainbow Spectra ELISA reader (Tecan US, Research Triangle Park, NC).

Transduction. NIH 3T3 cells (2.5×10^4) in 2 ml of D10 with Polybrene (8 μg/ml) were plated in each of the collagen-coated/virion-treated wells, and the cell cultures were incubated at 37°C in 5% CO_2 overnight. Cell culture medium was replaced with fresh D10 and the cultures further incubated for another 24 hr. Subsequently, cells were fixed and stained for the presence of β-galactosidase 48 hr after transduction.

The rat carotid injury model of vascular restenosis

Under general anesthesia (ketamine, 10 mg/kg; xylazine [Rompun], 5 mg/kg), in accordance with a protocol approved by the USC Institution Animal Care and Use Committee (IACUC), a 2F Intimax arterial embolectomy catheter (Applied Medical Resources, Laguna Hills, CA) was used to denude the carotid artery endothelium of Wistar rats (weights ranging from 375 to 425 g) as previously described (Zhu *et al.*, 1997). The catheter was inserted into the left external carotid artery, which was ligated distally, and passed into the left common carotid artery (LCCA). The balloon was inflated to a volume equiva-

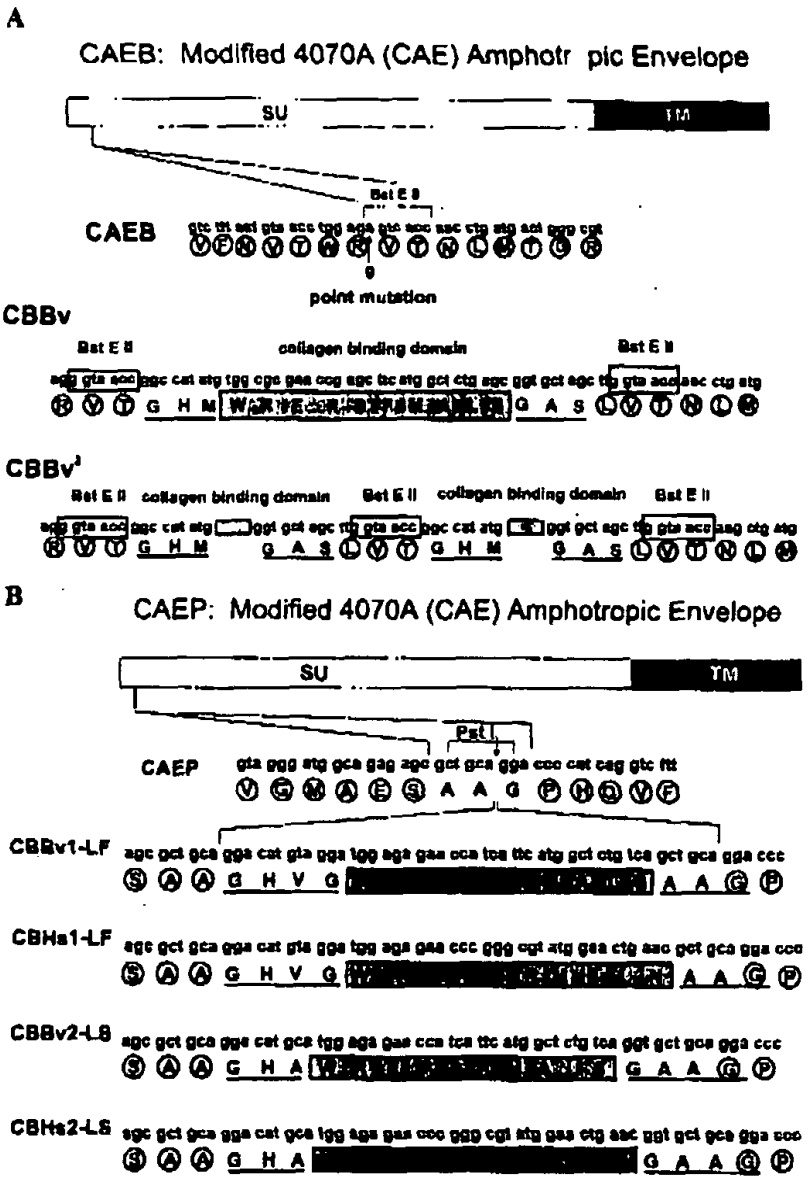

FIG. 2. Design and engineering of the collagen-targeted amphotropic Env proteins. (A) Schematic diagram of the modified envelope structure and cloning strategy used to insert the bovine collagen-binding decapeptide (WREPSFMALS) flanked by linker residues into the unique *Bst*EII site engineered by a single point mutation within the N-terminal region of the CAE Env protein. The underlined residues correspond to oligonucleotides used to generate the cDNA insert. (B) Schematic diagram of envelope structure and cloning strategy used to insert the WREPSFMALS (Bv) or the WREPGRMELN (Hs) collagen-binding decapeptide flanked by standard (LS) or special design (LF) linker residues into a unique *Pst*I site engineered within the N-terminal region of the CAE Env protein. The underlined residues correspond to oligonucleotides used to generate the respective cDNA inserts.

lent to 7F on a French scale cord, and passed three times along the length of the LCCA. After balloon injury, the embolectomy catheter was removed and the internal carotid artery was transiently ligated just distal to the bifurcation. Thirty microliters of either a collagen-targeted (CAEP/CBBv1-LF) or nontargeted (WT CAE) retroviral vector bearing a nuclear-targeted β-galactosidase construct with protamine sulfate (8 μg/ml) was instilled into the left common carotid artery immediately after balloon catheter injury or 7 days after injury. Seven days after vector instillation, the animals were killed and the arteries harvested

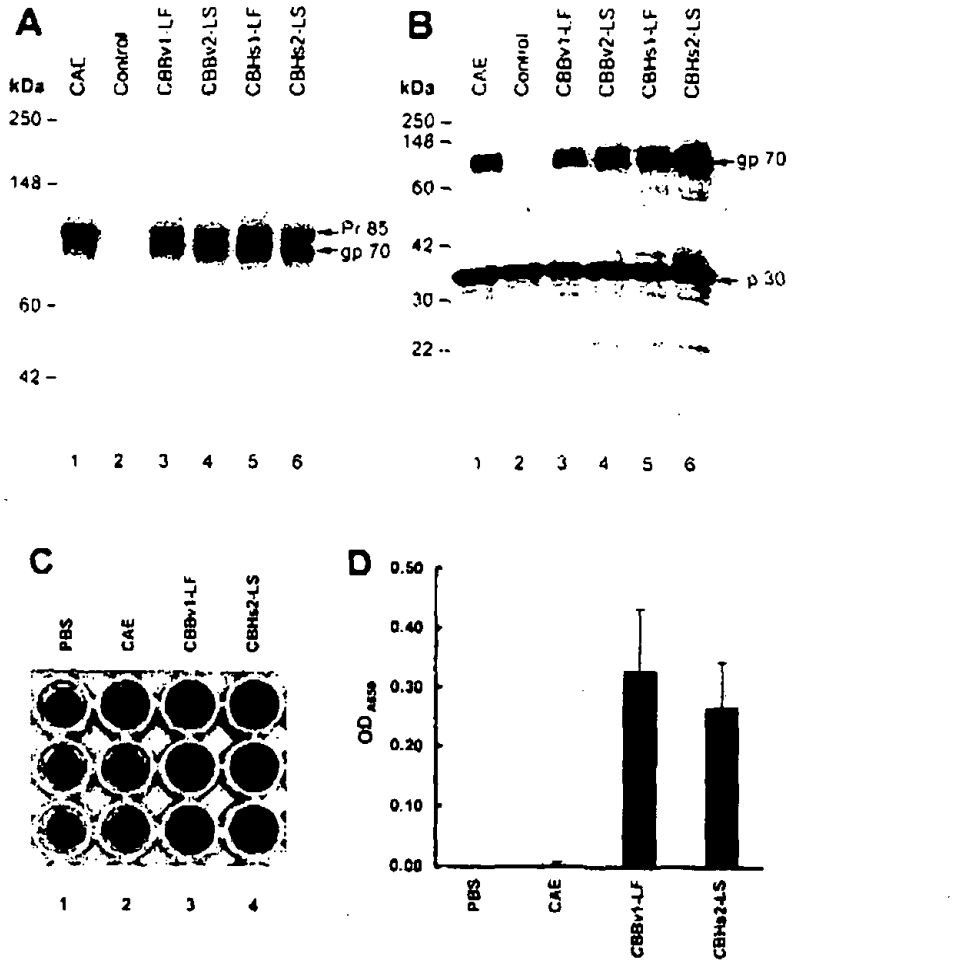

FIG. 3. (A) Expression of CBD-CAEP Env proteins in 293T cells. 293T cells were transfected with the specified Env protein expression plasmids (see Fig. 2), lysed 48 hr later in 500 μl of lysis buffer for 10 min, and centrifuged at 10,000 × *g* to pellet nuclei. The cell lysates were resolved on a precast gradient (8 to 16%) gel, and the processed SU subunit gp70^{env} proteins were detected by Western analysis, using a specific goat antiserum. (Zhu *et al.*, 1998). (B) Stable incorporation of CBD-CAEP Env proteins into viral particles. Western analysis using anti-gp70 and anti-p30 antisera shows incorporation of CBD-CAEP Env proteins into viral particles, as identified by antibodies directed against the viral Env protein fragments normalized to the p30 (*gag*) protein. (C and D) The conferred binding affinity of collagen-targeted vectors for collagen matrices. The comparative binding affinities of vectors bearing WT *env* (CEE or CAE) versus vectors displaying matrix-targeting motifs are inferred from the varying degrees to which ELISA wells are darkened (C), expressed as OD_{A650} readings on a Rainbow Spectra ELISA reader (D).

for hematoxylin–eosin (H&E) and X-Gal staining for the presence of nuclear β-galactosidase.

β-Galactosidase staining of rat carotid arterial segments

X-Gal solution was freshly prepared as follows: 1 ml of 0.2 *M* $K_4Fe(CN)_6 \cdot 3H_2O$, 1 ml of 0.2 *M* $K_3Fe(CN)_6$, and 0.08 ml of 1 *M* $MgCl_2$ were added to 1 × PBS to a total volume of 40 ml. The solution was incubated at 37°C for 30 min, and then mixed with 0.2 ml of prewarmed X-Gal solution (40 mg/ml). The harvested CCA was cut longitudinally, washed gently with 1 × PBS to remove the blood, and fixed in 3 ml of 10% formaldehyde for 1 hr at room temperature. The artery was then washed twice with 1 × PBS, placed in 2 ml of X-Gal staining solution, and incubated at 37°C overnight with gentle shaking. The artery was then washed with 1 × PBS, fixed in 10% formaldehyde for 15 min, embedded *in toto* in paraffin blocks, and processed according to standard operating procedures (university pathologists, Department of Pathology, University

of Southern California, Los Angeles, CA). Tissue sections were counterstained with nuclear fast red or hematoxylin-eosin stain.

A nude mouse model of liver metastasis

A total of 7×10^5 Miapaca cells (undifferentiated pancreatic cancer cells) were injected through the portal vein via an indwelling catheter, which was kept in place for 12 days. An infusion of 200 μl of either a nontargeted vector (WT CAE) or a collagen-targeted amphotropic retroviral vector (CAEP/CBBv1 LF) bearing a β-galactosidase construct was administered over 30 min starting on days 0, 3, 6, 9, and 12 after tumor cell infusion. The animals were killed on day 14, and the liver was harvested, fixed in 10% formaldehyde or quickly frozen in liquid nitrogen, and tissue sections stained with hematoxylin–eosin and X-Gal stain.

RESULTS AND DISCUSSION

The prototypical design of the matrix-targeted MuLV envelope protein involved the insertion of a bovine vWF-derived collagen-binding domain (see Fig. 1A) with strategic linkers (Hall *et al.*, 1997) into a unique *Bst*EII cloning site (between amino acids 18 and 19) of the CEE$^+$ ecotropic Env protein to produce the ECB-CEE Env, which is infectious with limited tropism (i.e., rodent-specific), and which exhibits a gain-of-function phenotype (i.e., high-affinity binding to collagen matrices). To extend vector tropism to human cells, we initially employed a dual envelope configuration (see Fig. 1B) in which a wild-type amphotropic Env (CAE, 4070A) is coexpressed and incorporated along with ECB-CEE Env into retroviral particles. To refine this design and eliminate steric hindrances resulting from the large inoperative binding domain of the ECB-CEE Env, we developed a series of targetable "escort" proteins in which the entire receptor-binding domain of the CEE$^+$ Env, from the *Bst*EII site to one of several unique (*Avr*II, *Stu*I, *Pst*I, or *Ngo*MI) sites engineered into the proline-rich hinge region (Wu *et al.*, 1998), was eliminated and replaced by the collagen-binding domain (see Fig. 1B). When a wild-type CAE Env was coexpressed with an Env construct bearing a matrix-targeting motif, the vectors arrayed in either the dual envelope configuration or the escort configuration exhibited high collagen-binding affinity (Fig. 1C and D) when compared with vectors bearing WT CEE or CAE *env* as determined by a modified ELISA (Hall *et al.*, 1997; Fig. 1D), as well as amphotropic infectivity, yielding wild-type titers of 10^7 CFU/ml in standard assays (Skotzko *et al.*, 1995). This is not surprising since many types of viruses stably express dual envelope configurations, such as hemagglutinin or distinct attachment glycoproteins, in addition to membrane fusion proteins. Hence, these compound configurations may be utilized in vector design to confer auxiliary targeting specificities. The escort configurations, in particular, may be suitable for insertion of large or bulky polypeptides, such as growth factors or antibody-binding motifs, i.e., protein A sequences (unpublished observations), into retroviral vectors.

Next, we engineered the integral gain-of-surveillance function into a single amphotropic envelope protein. Direct transposition of the matrix-targeting motifs from ecotropic (ECB-CEE) envelopes to a homologous site (*Bst*EII) engineered by point mutation within the amphotropic (CAE) *env* (Fig. 2A) yielded vectors that exhibited the collagen-binding phenotype provided by the addition of one (CBBv) or two (CBBv^2) bovine vWF-derived collagen-binding motifs (see Fig. 1C) but were devoid of infectivity when expressed as a single envelope. Thus, the structural constraints of the functional amphotropic and ecotropic Env proteins are distinct. Examination of the amino acid residues flanking the collagen-binding domain of vWF (see Fig. 1A) revealed a Pro-His "turn" or "kink" (Pro Arg in human) C terminal to the minimal CBD, a peculiarity that is also present at amino acids 7 and 8 in the CAE Env. Therefore, we engineered a structurally innocuous *Pst*I (Ala-Ala-Gly) cloning site into the CAE *env* at this position to generate CAEP (see Fig. 2B), into which a series of bovine or human vWF-derived matrix-targeting motifs flanked by flexible, glycine-rich linkers was inserted. As in the prototypical ECB-CEE design (Hall *et al.*, 1997), a histidine residue was included in the N-terminal linker to promote an external conformation of the collagen-binding domain. As shown in Fig. 3A, each of the resulting envelope proteins is readily expressed in human 293T producer cells, is correctly processed, and is incorporated equally as well as the WT CAE Env into retroviral particles (Fig. 3B). Each of the chimeric vectors bearing a collagen-binding motif exhibited significant collagen-binding affinity when compared with vectors bearing WT CEE or CAE *env* as determined by a modified ELISA (Hall *et al.*, 1997; Fig. 3C; $p < 0.05$). In contrast, the collagen-targeted vectors did not bind to plates coated with fibronectin, elastin, or laminin, indicating their specificity for collagen (data not shown). Remarkably, and most importantly, each of the resulting retroviral vectors remained infectious, yielding titers approaching those of vectors bearing WT CAE *env* ($\sim 10^7$ CFU/ml). As no major distinctions in the collagen-binding affinities or infectious titers of the CAEP series of Env constructs were discerned beyond minor batch-to-batch variations, one bovine (CBBv1-LF) and one human (CBHs2-LS) construct were arbitrarily selected for further study of performance *in vitro* and *in vivo*.

As shown in Fig. 4B, the matrix-targeted amphotropic vector (CBBv1-LF) bound to collagen-coated plates and remained infectious to overlaid human cells, while the vector bearing WT CAE *env* was washed away (Fig. 4A). The potential surveillance function of these matrix-targeted vectors was confirmed *in vivo*, using a rat carotid injury model described previously (Zhu *et al.*, 1997). On intraarterial instillation of the CAEP/CBBv1-F matrix-targeted vector immediately after balloon injury, increased transduction in focal areas of the treated arterial segment (up to 10%; $n = 2$; Fig. 5C and E) was observed when compared with animals treated with the nontargeted vector (<1%; Fig. 5B and D). An even greater increase in transduction efficiency was observed when the matrix-targeted vector was given 7 days after balloon injury (Fig. 6). Histological evaluation of the transduced arteries confirmed that the matrix-targeted vector enhanced the transduction of proliferative neointimal cells up to 20% in focal area within the treated arterial segment (Fig. 6C and D; $n = 2$) compared with <1% when using a vector with WT CAE *env* (Fig. 6A and B; $n = 2$). Taken together, these findings emphasize the therapeutic potential of deploying matrix-targeted retroviral vectors

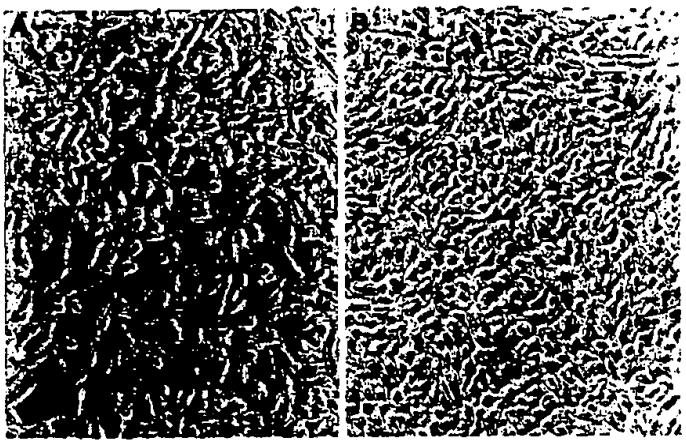

FIG. 4. (A and B) The binding affinity of collagen-targeted vectors for collagen matrices *in vitro* in a cell transduction assay. (A) No transduction of NIH 3T3 cells overlaid after washing with PBS was observed in wells treated with the retroviral vector bearing WT CAE *env*; (B) in contrast, appreciable transduction of NIH 3T3 cells overlaid on collagen-bound vectors bearing the CAEP/CBBv1 *env* was observed (cells with blue-staining nuclei).

for vascular restenosis, as well as coronary and peripheral artery diseases.

Since tumor invasion, as well as angiogenesis and stroma formation, evoke a remodeling of extracellular matrix componets and exposure of collagen (Stromblad and Cheresh, 1996), we advanced the hypothesis that retroviral vectors bearing an auxiliary matrix-targeting motif (i.e., collagen-binding polypeptides) would effectively concentrate at sites of newly exposed collagen in the vicinity of metastatic target cells. To assess the performance of these matrix-targeted vectors as tumor surveillance agents, a nude mouse model of liver metastasis was established by infusion of 7×10^5 pancreatic cancer cells into the portal vein via an indwelling catheter, which was kept in place for 14 days. Vector infusions consisting of 200 μl/day of either the targeted or nontargeted retroviral vector bearing a nuclear-targeted β-galactosidase marker gene (each

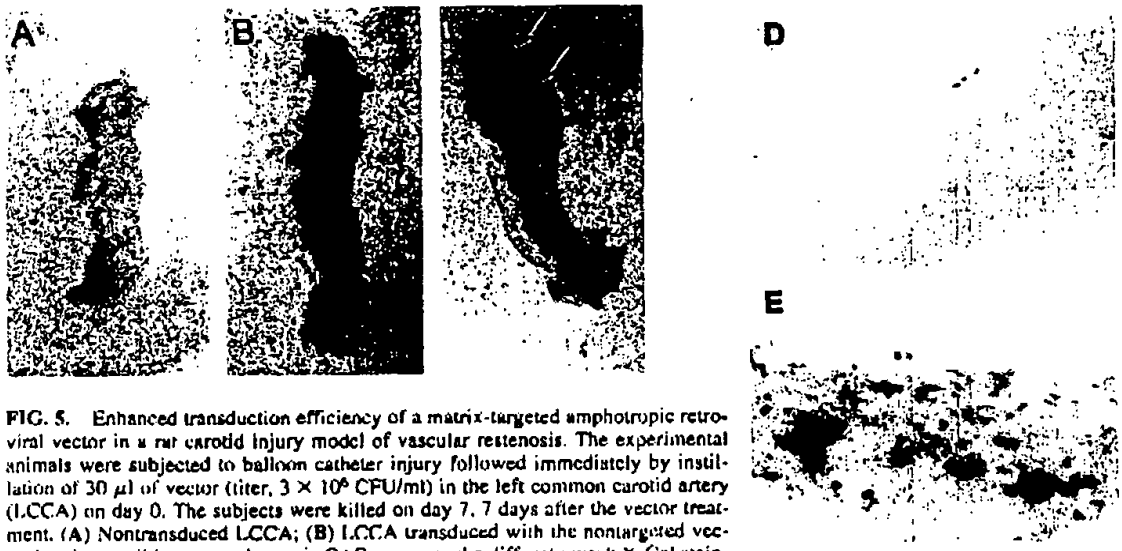

FIG. 5. Enhanced transduction efficiency of a matrix-targeted amphotropic retroviral vector in a rat carotid injury model of vascular restenosis. The experimental animals were subjected to balloon catheter injury followed immediately by instillation of 30 μl of vector (titer, 3×10^6 CFU/ml) in the left common carotid artery (LCCA) on day 0. The subjects were killed on day 7. 7 days after the vector treatment. (A) Nontransduced LCCA; (B) LCCA transduced with the nontargeted vector bearing a wild-type amphotropic CAE *env*; note the diffusely weak X-Gal staining throughout the LCCA; (C) LCCA transduced with a matrix-targeted vector (CAEP/CBBv1-LF). Note the intensely blue X-Gal staining of the LCCA, which represents the injured/transduced region (arrows); (D) arterial segment from (B) in paraffin block, showing a few β-galactosidase-positive cells (blue dots; original magnification, $\times$200; (E) arterial segment from (C) in paraffin block, showing β-galactosidase-expressing cells (blue dots; original magnification, $\times$200).

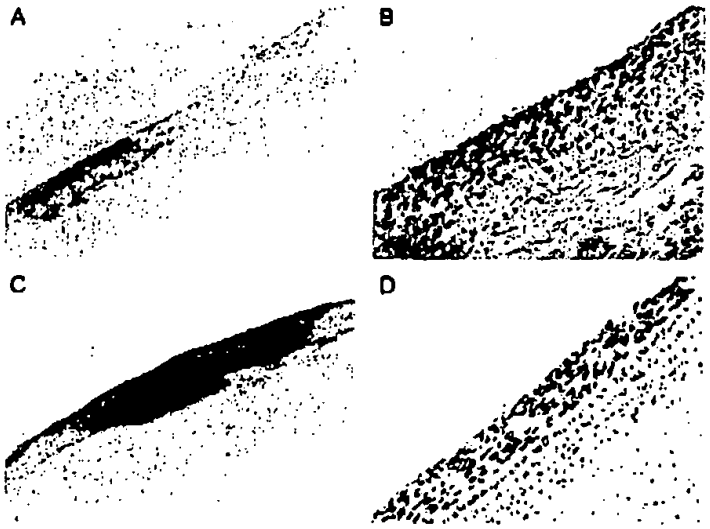

FIG. 6. Enhanced transduction efficiency of matrix-targeted amphotropic retroviral vectors in a rat carotid injury model of vascular restenosis. The experimental animals were subjected to balloon catheter injury on day 0. On day 7, 30 μl of vector (titer, 2×10^7 CFU/ml) was instilled into the LCCA. The animals were killed 7 days after vector treatment (day 14), and the harvested LCCAs stained with X-Gal stain. (A) LCCA transduced with the nontargeted vector bearing a wild-type amphotropic CAE *env*; note the diffusely weak X-Gal staining throughout the LCCA; (B) histological section of (A), showing a few β-galactosidase-positive neointimal cells with blue-staining nuclei (original magnification, ×200); (C) LCCA transduced with a matrix-targeted vector (CAEP/CBBv1-LF). Note the intensely blue X-Gal staining of the LCCA, which represents the injured/transduced region. (D) Histological section of (C), showing the β-galactosidase-positive neointimal cells (original magnification, ×200).

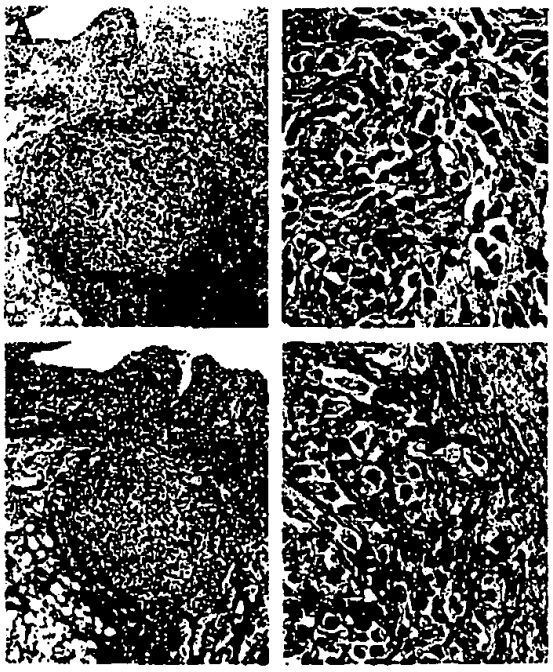

FIG. 7. Matrix-targeted retroviral vectors deployed in pursuit of metastatic cancer. Transduction of a tumor nodule in a nude mouse model of liver metastasis. β-Galactosidase-positive tumor cells (cells with blue-staining nuclei) are shown within a metastatic tumor nodule *in situ*. (A) X-Gal stain plus H&E stain; (B) higher magnification of (A); (C) X-Gal stain plus smooth muscle actin stain; (D) higher magnification of (C). Original magnification: (A and C) ×40; (B and D) ×400.

vector titer, 1×10^7 CFU/ml) were given at 3-day intervals for a total of five infusions, and the mice were killed 2 days after completion of the vector infusions. Histopathologic examination of the livers from control animals revealed abundant tumor foci with attendant areas of angiogenesis and stroma formation. As seen in Fig. 7, we observed focal areas of transduction of tumor cells *in vivo* within the metastatic tumor foci (1–3%; $n = 6$), as evidenced by the presence of β-galactosidase-positive tumor cells (cells with blue-staining nuclei). In contrast, no definitive transduction was observed in metastatic lesions in mice treated with nontargeted vectors, nor was transduction observed in the liver parenchyma itself. Considering the overall mass of the liver and the physiological dilution factors and surface areas introduced by intravascular injection, these findings represent a considerable advancement in the requisite technology for deploying targeted injectable retroviral vectors in pursuit of metastatic cancer.

Although several creative systems for targeting retroviral vectors have been designed (Cosset and Russell, 1996; Peng *et al.*, 1997), ECM targeting remains among the most promising approaches developed to date (Anderson, 1998). In one application utilizing fibronectin, which is present in normal ECM, enhanced retroviral transduction of hematopoietic cells has been achieved (Moritz *et al.*, 1996), and is based on the observation that retroviruses can bind nonspecifically to fibronectin and thus be colocalized with target cells. However, this approach cannot be used for *in vivo* targeting. In contrast, targeting of retroviral vectors by incorporation of a surveillance function of von Willebrand factor (i.e., a high affinity for collagen) represents a major step toward the development of a targeted injectable vector for intravascular gene delivery (Salmons and Gunzburg, 1993; Anderson, 1998). The potential clinical utility of this extracellular matrix-targeting strategy extends well beyond hemostasis and vascular injury (i.e., restenosis and ischemia) to a number of clinical situations in which ECM (i.e., collagen) is characteristically exposed, including laser and other surgical procedures, orthopedic injuries, wound healing, arthritis, inflammatory disease, and metastatic cancer. In conclusion, the present study extends the concept to ECM targeting from ecotropic to amphotropic vectors and, thus, the potential applications of these targeted injectable retroviral vectors for human gene therapy.

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Address reprint requests to:
Dr. Erlinda M. Gordon
Gene Therapy Laboratories
1975 Zonal Avenue, KAM 300
Los Angeles, CA 90089-9025

E-mail: emgordon@genome2.hsc.usc.edu

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NONVIRAL TRANSFER TECHNOLOGY RESEARCH ARTICLE

Development of a hypoxia-responsive vector for tumor-specific gene therapy

T Shibata, AJ Giaccia and JM Brown
Mayer Cancer Biology Research Laboratory, Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305-5468, USA

We are developing new gene therapy vectors whose expression is selectively activated by hypoxia, a unique feature of human solid tumors. As vascular endothelial growth factor (VEGF) is upregulated by hypoxia, such regulatory mechanisms would enable us to achieve hypoxia-inducible expression of therapeutic genes. Constructs with five copies of hypoxia-responsive elements (HREs) derived from the 5'-untranslated region (UTR) of the human VEGF showed excellent transcriptional activation at low oxygen tension relevant to tumor hypoxia. In an attempt to achieve higher responsiveness, various combinations of HREs and promoters were examined. In addition, we also investigated whether the 3' UTR of the VEGF gene would confer increased post-transcriptional mRNA stability under hypoxic conditions. However, despite increases in the hypoxic/aerobic ratio of luciferase activity, gene expression with 3' UTR was lower due to mRNA destabilization by AU-rich elements (AREs). Thus, we found no benefit from the inclusion of the 3' UTR in our vectors. Of all the vectors tested, the combination of 5HRE and a CMV minimal promoter exhibited hypoxia responsiveness (over 500-fold) to the similar level to the intact CMV promoter. We propose that this vector would be useful for tumor selective gene therapy. Gene Therapy (2000) 7, 493–498.

Keywords: *tumor hypoxia; hypoxia-responsive element; hypoxia-inducible factor-1; gene therapy; vascular endothelial growth factor*

Introduction

A fundamental problem for cancer gene therapy is the lack of a tumor-selective delivery system. One approach to overcome this, at least in part, is to develop tumor specific gene expression. At present, preclinical studies are underway to investigate the utility of tumor- or tissue-specific promoters such as prostate-specific antigen promoters for prostate cancers,[1] carcinoembryonic antigen promoters for colorectal cancers,[2] and erbB2 promoters for breast cancers.[3] Recent studies have also explored the potential of exploiting tumor hypoxia as a gene therapy target.[4–7]

Hypoxia, a unique feature of human solid tumors,[8] has been considered to be a major factor in the resistance of cancers to radiotherapy and chemotherapy. Recent clinical studies using oxygen electrodes have provided compelling evidence that human tumors contain regions at low oxygen partial pressure, and that the more hypoxic tumors have a poor prognosis after treatment.[9–11] Besides these effects on treatment outcome, hypoxia is a potent signal inducing the expression of a number of genes including erythropoietin (Epo), VEGF, and various glycolytic enzymes.[12–14] Hypoxia responsible elements (HREs) have been reported in the 3' flanking region of the human and mouse Epo genes, and a hypoxia-inducible factor 1 (HIF-1) has been shown to bind to the consensus sequence in the Epo HRE.[15,16] Similar HIF-1 binding sequences have been found in the 5' flanking region of other hypoxia inducible genes including VEGF.[17–19] Such regulatory mechanisms for hypoxia responsive gene expression could provide a means for tumor-specific therapeutic approaches. Dachs *et al*[6] have demonstrated that heterologous gene expression driven by HREs from the mouse phosphoglycerate kinase-1 (PGK-1) gene could be activated in hypoxic tumor cells. We have previously shown that a vector with five copies of HRE and a E1b minimal promoter exhibited a 40- to 50-fold increase in gene expression under hypoxia when transiently transfected into human tumor cells.[7] However, despite its robust responsiveness under hypoxia, the amount of luciferase activity for this vector was considerably smaller than that produced by a CMV promoter vector, and may be insufficient to achieve gene expression for genetically directed enzyme prodrug therapy (GDEPT). We have therefore carried out further studies with the goal of establishing hypoxia-inducible vectors with higher expression of therapeutic genes.

In addition to transcriptional activation via HIF-1 binding to HREs, post-transcriptional regulation of the VEGF mRNA stability may be an important factor for up-regulation of VEGF expression under hypoxia. Recent studies in an *in vitro* RNA degradation assay revealed that some genetic elements for mRNA stability are located in the 3' UTR.[20–22] The VEGF 3' UTR contains a cluster of AU-rich elements (ARE) including the AUUUA pentamer and the UUAUUUAUU nonamer, as do many labile mRNAs encoding lymphokines, cytokines, transcriptional factors,

Correspondence: JM Brown
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and proto-oncogenes.[23-25] Five hypoxia-inducible RNA protein-binding sites have been identified in human and rat genes that might regulate mRNA stability under hypoxia.[22,26] However, the usefulness of such post-transcriptional mechanisms in hypoxia-inducible vectors is not clear. Levy *et al*[26] reported that transfer of the rat VEGF 3' UTR to a heterologous gene does not confer hypoxia-inducible mRNA stability on the heterologous transcript. However, Damert *et al*[27] have published that a reporter vector with the 3' UTR of the mouse VEGF gene produced a marked increase in β-galactosidase activity within experimental gliomas, suggesting that an increase in mRNA stability may contribute to VEGF induction under hypoxic conditions. Such different regulatory mechanisms may provide the potential of exploiting tumor hypoxia for targeted gene expression.

To characterize further the transcriptional activation capacity of HREs from human VEGF under hypoxic conditions, we examined the aerobic and hypoxic responsiveness of luciferase reporter genes as a function of oxygen concentration. We also examined whether post-transcriptional mRNA stabilization by VEGF 3' UTR could improve hypoxia inducibility. In order to increase the hypoxia inducible level of gene expression, we tested various constructs to determine the most optimal combination of HREs and several promoter elements. We found that a construct composed of 5HRE ligated to a human CMV minimal promoter increased gene expression over 500-fold in response to hypoxia, reaching levels comparable to that obtained by the full length CMV IE promoter. This vector, therefore, may be useful in an appropriate vehicle to obtain tumor-specific gene expression by exploiting tumor hypoxia.

Results

Oxygen dependent inducibility of a hypoxia-inducible vector with 5HRE

In our previous study, a 5HRE/VEGF/E1b vector showed a significant increase in luciferase expression in transient transfected human tumors after 6 h of hypoxic exposure. Because human tumors contain cells at various oxygen concentrations, we measured transcriptional induction of our 5HRE reporter gene as a function of oxygen concentration. HT1080 cells were transfected with 5 μg of vector DNA per 5×10^5 cells, exposed to various oxygen concentrations between 21% and 0.02% for 6 h, and assayed for luciferase activity. As shown in the Figure 1, a significant increase in luciferase activity was detected below 2% oxygen and continued to increase as oxygen levels decreased, as reported earlier.[28] For the 5HRE/VEGF/E1b vector, a 40- to 50-fold increase was observed after 6 h hypoxic treatment at 0.02% O_2. These data suggested that hypoxia mediated transcriptional activation could be achieved under oxygen levels relevant to those in human solid tumors.

Effects of copy number of HREs on hypoxia inducible gene expression

We next examined the effect of increasing HRE copy number of HREs on the hypoxia inducibility of the luciferase reporter gene. A series of the reporter vectors with three to 10 copies of HRE ligated to the SV40 promoter derived from the pGL3 vector or the E1b minimal promoter were generated as depicted in the Figure 2. For all the pGL3 vector constructs with HREs, significant increases in luciferase activity were observed after 6 h of hypoxic treatment at 0.02% O_2. The hypoxic/aerobic ratios ranged about 20–60-fold and increased with the numbers of HREs. However, a saturation effect was observed for vectors with more than five copies of HREs.

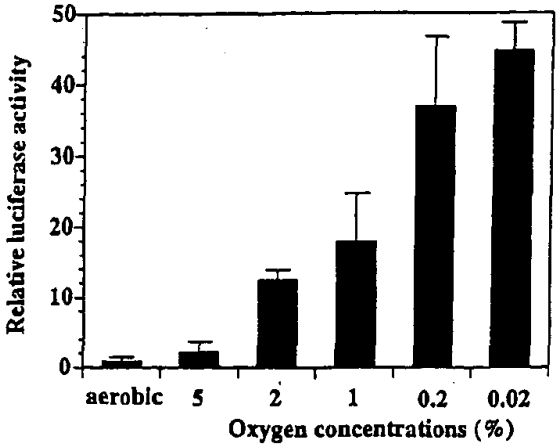

Figure 1 The luciferase activity of a 5HRE/VEGF/E1b vector under aerobic and hypoxic conditions as a function of oxygen concentration. HT1080 cells were transfected with vector DNA (5 μg per 5×10^5 cells), the cells were divided into the glass culture dishes, allowed to recover for 36 h after transfection under aerobic conditions, exposed to various oxygen concentrations for 6 h, and assayed for luciferase activity. The luciferase activities were normalized to that under aerobic conditions. The error bars in all the Figures show the standard deviation (s.d.) of at least four independent samples.

We also compared the hypoxia responsiveness of constructs with five and 10 copies of HREs in a series of reporter genes with an E1b minimal promoter. The hypoxic/aerobic ratios for both vectors were about 40 and 60 at 0.2 and 0.02% O_2, respectively. Thus, five copies of HREs ligated to either basal promoter results in maximal hypoxia inducible expression.

The effect of the VEGF 3' UTR on reporter gene inducibility

Recent studies have suggested that VEGF mRNA could be stabilized post-transcriptionally under hypoxia through its 3' UTR sequences.[20,22,26] Thus, the VEGF 3' UTR could potentially enhance hypoxia responsiveness of HRE regulated gene constructs by increasing mRNA stability. To obtain 3' UTR sequences, HT1080 cells were treated under hypoxia for 24 h, total RNAs was isolated, and RT-PCR was performed as described in Materials and methods. As 1.5 kb of the 3' UTR fragment from this study encompasses all five hypoxia-inducible RNA protein binding sites reported by Levy *et al*[26] we ligated this fragment to the pGL3 and 5HRE/VEGF/E1b vectors as shown in Figure 3.

HT1080 cells were transfected with the pGL3 and 5HRE/VEGF/E1b vectors with or without 1.5 kb of the 3' UTR, allowed to recover for 36 h after transfection, exposed to 0.02% O_2 for 6–18 h, and assayed for luciferase activity. Significant increases in luciferase activity were detected after hypoxic treatment for the pGL3–3' UTR

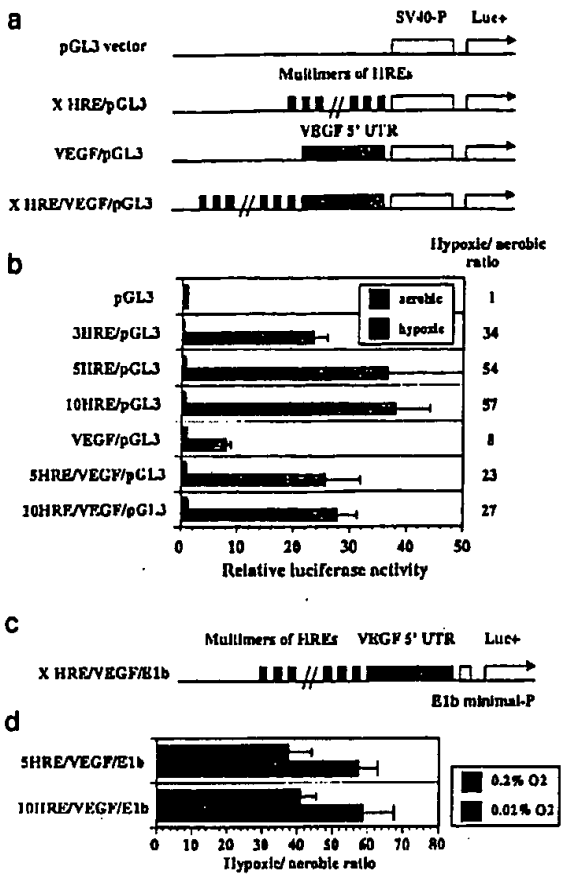

Figure 2 Effect of HRE dosage on hypoxia responsiveness for pGL3 promoter vectors and E1b minimal promoter vectors. HT1080 cells transfected with vectors were divided into the glass culture dishes, allowed to recover for 36 h, and treated under hypoxia (0.02% O_2) for 6 h. (a) Diagram of pGL3 promoter vectors with multimers of HREs and a 318 bp of VEGF 5' UTR fragment. (b) The luciferase activity of pGL3 promoter vector series. Each column represents relative activity (mean and s.d.) to that for pGL3 vector under aerobic condition and hypoxic/aerobic ratio are indicated. (c) Diagram of E1b minimal promoter vector series. (d) The hypoxic/aerobic ratios are shown for 5- and 10HRE/VEGF/E1b vectors at 0.2 and 0.02% O_2.

vector in comparison with its aerobic control, while significant decreases were observed after 18 h hypoxia for the pGL3 vector. Although the addition of the 3' UTR resulted in moderate increases (1.5-fold) in the hypoxic/aerobic ratios, the actual amount of luciferase activity for the pGL3–3' UTR were smaller than that for the pGL3 vector.

Similarly, the hypoxic/aerobic ratios of approximately 200-fold that were obtained with the 3' UTR for the 5HRE/VEGF/E1b series were mainly due to reduced luciferase activity under aerobic conditions. In fact, the actual amount of expression of reporter genes with 3' UTR was smaller than reporter genes without the 3' UTR under hypoxia. Thus, the 3' UTR of the human VEGF gene produced both a marked destabilization under aerobic conditions and increased stability under hypoxia.

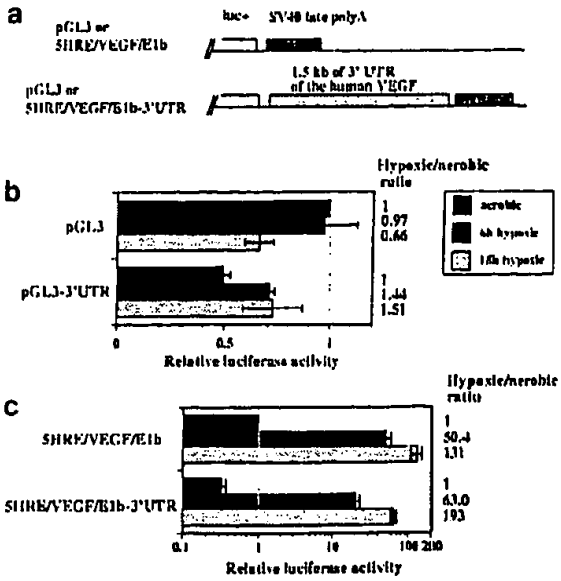

Figure 3 Effect of the VEGF 3' UTR on aerobic and hypoxic responsiveness. (a) Diagram of pGL3 and 5HRE/VEGF/E1b vectors with or without 1.5 kb of the VEGF 3' UTR. (b) The luciferase activity for pGL3 and pGL3–3' UTR vectors. After transfections of vectors, HT1080 cells were treated under hypoxic conditions for 6–18 h. Each column shows relative activity (mean and s.d.) to that for pGL3 under aerobic conditions. (c) The luciferase activity for 5HRE/VEGF/E1b and 5HRE/VEGF/E1b-3' UTR vectors. Each column shows relative activity (mean and s.d.) to that for 5HRE/VEGF/E1b under aerobic conditions.

Use of the CMV minimal promoter combined with HREs

Although the 5HRE exhibited robust responsiveness under hypoxia, the actual amount of luciferase activity for the 5HRE/VEGF/E1b vector was about 100-fold lower than that for a CMV IE promoter-driven vector. As a reporter gene, the 5HRE/VEGF/E1b vector possessed low background activity in air and high responsiveness under hypoxia. However, even with maximum induction by hypoxia, it is unclear whether this would be effective in CDEPT because anti-tumor effects will likely depend critically on the actual amount of the prodrug activating enzyme. We therefore examined other constructs with the goal of achieving higher absolute expression levels under hypoxia. Two such constructs were derived from the human EF-1 alpha promoter, one of the strongest mammalian promoters, and a 60 bp minimal promoter containing a TATA sequence derived from the human CMV IE promoter.

As shown in Figure 4, there was undetectable hypoxic induction with the 5HRE/EF-1 alpha vector. In fact, after 18 h hypoxic treatment at 0.02% O_2, luciferase activity was markedly reduced. However, with the 5HRE/hCMVmp vector, we could detect more than a 500-fold induction by 18 h hypoxia at 0.02% O_2. Both absolute levels of gene expression were high (about 0.4 of CMV I/E), and basal activity was slightly above background empty vector levels (less than 0.001 of CMV I/E). Also shown (Figure 4c) is the oxygen dependence for induction by this vector. The data show a similar oxygen dependence for inducibility as the 5HRE/VEGF/E1b construct but with a larger dynamic range. Thus, among

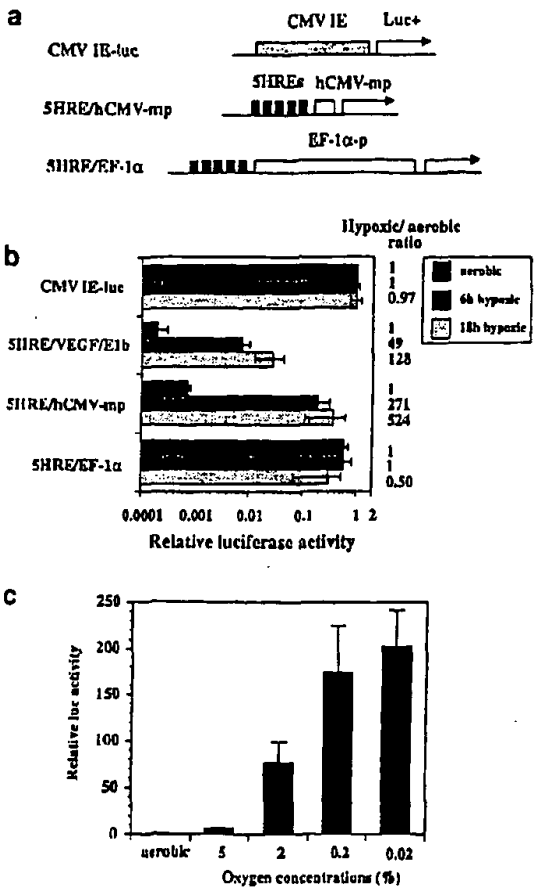

Figure 4 Comparison of 5HRE with different promoter elements. (a) Diagram of various combination of the HREs and promoters. (b) Comparison of the hypoxia inducibility for each vector. The luciferase activities are shown as relative values (mean and s.d.) to that for CMV IE vector under aerobic conditions. Hypoxic/aerobic ratios are also indicated. (c) The luciferase activity of the 5HRE/hCMVmp vector as a function of oxygen concentration. The procedures were the same as described in Figure 1.

all the constructs tested, the 5HRE/hCMVmp appears to be the most promising vector that could be utilized in hypoxia-inducible gene therapy.

Discussion

We and others have proposed that the lower level of oxygenation found in solid tumors compared with normal tissues may represent a means to target gene expression selectively to tumors.[6-8,29] However, to date, there have been no reports of successful implementation of this strategy. Possible reasons include the need for levels of hypoxia/anoxia that are lower than those occurring in tumors, and/or inadequate specificity or potency of the vectors and therapeutic genes used. We have addressed these specific questions in the present studies.

Our results on the oxygen dependency of gene induction (Figure 1) suggest that the 5HRE-derived constructs give maximum gene expression in the human tumor cells at an oxygen concentration of 0.2% (1.5 mmHg) and half-maximum activity at 1% oxygen concentration (5–7 mmHg). This finding is similar to that reported earlier for HIF-1 DNA-binding activity in HeLa cells, where a maximum response was obtained at 0.5% oxygen and a half-maximum at about 1.5% oxygen.[28] In several clinical series the average median pO_2 for carcinomas of the breast, head and neck, and cervix are 10–15 mmHg, with most tumors having areas with pO_2 values <5 mmHg.[9-11] Further, we have recently shown that transplanted tumors in mice have even lower levels of oxygenation with average median values of 1–3 mmHg.[30] Thus, the activation of hypoxia-responsive genes acting through HIF-1 binding to HREs is in the ideal range for selective gene expression in human and experimental tumors.

The second question as to selectivity and potency deals with both relative and absolute expression under hypoxia. An earlier study of Dachs *et al*[6] using mouse PGK, TK, and 9–27 promoters with HREs driving a cytosine deaminase gene in stably transfected cells reported a 5.4-fold sensitization to 5-FC after 16 h hypoxia. Using a luciferase reporter assay following transient transfection we reported 40- to 50-fold increases in hypoxia induction with a construct containing five copies of a 35-bp fragment derived from the VEGF HRE and a 32-bp fragment of the E1b minimal promoter sequence.[7] However, maximum expression by this construct was some 100-fold lower than that achieved with a strong, constitutive promoter such as the cytomegalovirus (CMV) immediate/early promoter. In the present investigation we sought to increase both the specificity (relative hypoxia/aerobic expression) as well as the potency (absolute expression under hypoxia) of the hypoxia-responsive vector.

Our first attempt was to use the fact that increased post-transcriptional stability of mRNA plays an important role in increased VEGF expression under hypoxic conditions. Several authors have reported that the half-life of VEGF mRNA is increased 2.5- to eight-fold in response to hypoxia.[20-22] Damert *et al*[27] reported that stably transfected cells with *LacZ* reporter genes that possessed 3′ UTR sequences of the mouse VEGF gene showed positive staining for beta-galactosidase in the perinecrotic areas in transplanted tumors, suggesting that these sequences provide increased stability under hypoxia. Also a recent study by Claffey *et al*[31] showed that chimeric reporters containing VEGF 3′ UTR sequences between the luciferase coding sequence and the polyadenylation site were significantly increased by 12 h hypoxic incubation. Levy *et al*[26] identified five potential hypoxia-inducible protein binding sites by RNA electromobility shift assay in the human and rat 3′ UTR, providing a regulatory mechanism for mRNA stability. However, contrary to previous reports, we found that under hypoxia the luciferase expression with 3′ UTR was lower than that without these sequences under hypoxia even though the hypoxic/aerobic ratio was significantly increased with the 3′ UTR sequences. The reason for this is suggested by the fact that the VEGF 3′ UTR contains a cluster of AU repeats similar to that in many labile and rapid turnover mRNAs including those of cytokines, transcription factors and proto-oncogenes. Insertion of AREs from the 3′ UTR of granulocyte–macrophage colony-stimulating factor or c-fos has been also shown to destabilize a normally stable globin mRNA.[25,32] Thus, we believe that the addition of the 3′ UTR from VEGF acted

to destabilize the message under aerobic conditions, and this was partially reversed under hypoxia by putative hypoxia-induced RNA binding proteins. The result of this is that there was no advantage to adding the 3' UTR to the reporter construct.

As noted above, the amount of gene expression with 5HRE/VEGF/E1b had an excellent hypoxia/aerobic ratio of approximately 100. However, the absolute levels of expression even with prolonged hypoxia were still approximately 100-fold lower than with a CMV-driven vector. Therefore, we tested the highly potent human EF-1 alpha promoter with 5HRE, but although this plasmid construct produced high levels of aerobic expression, there was no hypoxia inducibility (Figure 4). When we replaced the E1b minimal promoter with a minimal sequence of the CMV IE promoter, we found an increased expression of over 500-fold by an 18 h hypoxia treatment. Importantly, this 5HRE/hCMVmp construct produced a level of expression that was almost equivalent to that produced by the full-length CMV IE promoter (Figure 4). Similar minimal promoters derived from the CMV promoter have been used with multimers of tetracycline-responsive operators originally reported by Gossen *et al*[33] and more than a 1000-fold induction of reporter gene expression has been achieved by addition of doxycycline for 24 h.[34] CMV minimal promoters were also activated to a significantly higher degree than a thymidine kinase minimal promoter. Thus, the choice of minimal promoters seems to be important, and the use of a CMV minimal promoter may be the best for transcriptional targeting strategies.

The present study provides a significant advance in hypoxia-selective gene expression than presently available. We believe that such constructs can be used to drive therapeutic genes such as enzymes that can metabolize nontoxic prodrugs into toxic drugs. The selective expression of these enzymes in the tumors will, therefore, allow tumor specific activation of these toxic drugs by the poorly oxygenated regions of solid tumors. *In vivo* experiments to test this concept are currently under way.

Materials and methods

Cell culture and hypoxic treatment

HT1080 cells were obtained from the American Type Culture Collection, Rockville, MD, USA and cultured in alpha-MEM with 10% FCS in a well-humidified incubator with 5% CO_2 at 37°C. For transient transfection experiments, 5×10^5 exponentially growing cells were plated on a six-well culture dish overnight as described previously.[7] Hypoxic conditions were achieved using pre-warmed aluminum hypoxic chambers[35] by evacuation and gassing with 95% N_2/5% CO_2, and then the tightly sealed chambers were incubated at 37°C for 6–18 h. The oxygen concentrations were controlled to be the desired levels from 2% to 0.02% as described previously.[35]

Plasmid construction, transfection and reporter assay

The methods for construction of a 5HRE/VEGF/E1b vector and preparation of the multimers of HREs were previously described.[7] Schematic diagrams for each construct are shown in each Figure. To search for an optimal construction of a vector having large hypoxia inducibility, we generated a series of luciferase reporter plasmids by combinations of the following gene fragments into the pGL3 vector (Promega, Madison, WI, USA): the 385 bp fragment of the VEGF gene derived from the 5' flanking region at position −1175 to −790 from the transcription start site[36] and the multiple copies of a HIF-1 consensus sequence of the human VEGF gene.[7,37] To make a 5HRE/EF-1a vector, an *EcoRI–XhoI* fragment from a pEF/myc/cyto vector (Invitrogen, Carlsbad, CA, USA) was cloned into a pcDNA3 vector (Invitrogen) and a human EF-1a promoter prepared as a *BamHI–NcoI* fragment was inserted into a *BglII–NcoI* site of the 5HRE/pGL3 vector. A synthetic fragment consisted of a human cytomegalovirus minimal promoter (hCMVmp) and linker sequences was also inserted at a *BglII–HindIII* site of 5HRE/pGL3 for making a 5HRE/hCMVmp vector. The hCMVmp using in this study encompasses the sequence between −53 and +7 of the original promoter sequence reported previously.[38]

To examine the effects on hypoxia responsiveness via a putative mRNA stability mechanism, we added a fragment from the 3' UTR of the human VEGF gene behind the luciferase gene. The 1.5 kb of 3' UTR were obtained by RT-PCR.[26] Total RNA of HT1080 cells after 24 h hypoxic treatment were extracted by RNeasy kit (Qiagen, Santa Clarita, CA, USA) and the first-strand templates were prepared by Superscript reverse transcription system (Gibco BRL, Rockville, MD, USA). PCR was performed using Elongase enzyme mixture (Gibco BRL) with the following paired primers: 5'-CGCTCTAGATAGACACACCCACCCACATA-3' and 5'-GAGAGCTAGCCTACGGAATATCTCGAAAAACTGCAC-3'. The PCR conditions were 94°C, 1 min; 52°C, 1 min; 68°C, 2 min; for 35 cycles. The fragment were inserted at *XbaI* site of pGL3 and 5HRE/VEGF/E1b vectors.

For transient transfection assays, these test plasmids and the control pRL-CMV plasmid were co-transfected into tumor cells with the Superfect reagent (Qiagen) followed by incubation for 3 h. The culture dishes were washed with PBS twice and fresh growth media added. At 36 h after transfection, the cells were trypsinized and plated into the notched glass dishes overnight before the hypoxic treatment. After 2 h of reoxygenation, cell lysates were prepared with 400 µl of passive lysis buffer using a Dual Luciferase Assay kit (Promega) and the luciferase activities were measured by a luminometer. To minimize the variations of transfection efficiency between experiments, we performed dual luciferase measurements and normalized the luciferase activities of test plasmids with those of the control plasmids as described previously.[7] In experiments comparing the actual amount of gene expression among various promoters, protein concentrations of the cell lysates were also determined by Bradford's method (BioRad, Hercules, CA, USA).

Acknowledgements

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Long-term survival in a rodent brain tumor model by bradykinin-enhanced intra-arterial delivery of a therapeutic herpes simplex virus vector.

Rainov NG, Dobberstein KU, Heidecke V, Dorant U, Chase M, Kramm CM, Chiocca EA, Breakefield XO.

Department of Neurosurgery, Faculty of Medicine, Martin Luther University, Halle, Germany.

Recently, it was demonstrated that bradykinin (BK) enhances intracarotid delivery of herpes simplex virus type I (HSV) vectors to rat brain tumors, and that gene transfer takes place predominantly in the tumor periphery. The aim of the present study was to apply these findings to the treatment of experimental rat brain tumors. The HSV mutant, hrR3, which is disrupted in the ribonucleotide reductase gene, was injected intra-arterially with titers of 1 x 10(8), 1 X 10(9), and 1 x 10(10) plaque-forming units (pfu) both with and without BK into Fischer 344 rats with intracerebral, syngeneic 9L tumors. Starting on day 3 after vector administration, animals were treated by intraperitoneal injection of 60 mg/kg/day ganciclovir (GCV) or placebo. 1 x 10(10) pfu hrR3 in combination with BK and GCV treatment was able to eradicate tumors in 80% of the animals; 1 x 10(9) pfu cured 40% of the rats, and 1 x 10(8) pfu achieved an extension of survival time but no tumor cures. Control groups had 100% mortality within 30 days after injection of tumor cells, with the exception of the group with injection of 1 x 10(10) pfu of virus and GCV treatment, which had one long-term survivor. No apparent complications of this novel type of brain tumor gene therapy were encountered. In conclusion, intra-arterial injection of attenuated HSV vectors with blood-tumor barrier modification and subsequent systemic GCV application appears to be a promising approach for the treatment of malignant brain tumors.

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Mary Ann Liebert, Inc.

Intraarterial Delivery of Adenovirus Vectors and Liposome–DNA Complexes to Experimental Brain Neoplasms

NIKOLAI G. RAINOV,[1,2] KEIRO IKEDA,[3] NAZIR H. QURESHI,[3] SHIVANI GROVER,[1] ULRICH HERRLINGER,[1] PETER PECHAN,[1,4] E. ANTONIO CHIOCCA,[3] XANDRA O. BREAKEFIELD,[1] and FAITH H. BARNETT[1,5,6]

ABSTRACT

This study investigated the intraarterial delivery of genetically engineered replication-deficient adenovirus vectors (AVs) and cationic liposome–plasmid DNA complexes (lipoDNA) to experimental brain tumors. Adenovirus or lipoDNA was injected into the internal carotid artery (ICA) of F344 rats harboring intracerebral 9L gliosarcomas, using bradykinin (BK) to selectively permeabilize the blood–tumor barrier (BTB). Brain and internal organs of the animals were collected 48 hr after vector injection and stained for expression of the marker gene product, β-galactosidase (β-Gal). *Intracarotid delivery of AV to 9L rat gliosarcoma without BTB disruption resulted in transgene expression in 3–10% of tumor cells distributed throughout the tumor.* Virus-mediated expression of β-*gal* gene products in this tumor model was particularly high in small foci (≤0.5 mm), which had invaded the normal brain tissue surrounding the main tumor mass. In these foci more than 50% of tumor cells were transduced. BK infusion increased the amount of transgene-expressing cells in larger tumor foci to 15–30%. In the brain parenchyma only a few endothelial cells expressed β-*gal* owing to AV-mediated gene transfer. Intracarotid delivery of lipoDNA bearing a cytoplasmic expression cassette rendered more than 30% of the tumor cells positive for the marker gene without BTB disruption. The pattern of distribution was in general homogeneous throughout the tumor. BK infusion was able to increase further the number of transduced tumor cells to more than 50%. Although lipoDNA-mediated gene transfer showed increased efficacy as compared with AV-mediated gene transfer, it had less specificity since a larger number of endothelial and glial cells also expressed the transgene. AV and lipoDNA injections, in the absence and presence of BK, also resulted in transduction of peripheral organs. AV showed its known predilection for liver and lung. In the case of lipoDNA, parenchymal organs such as liver, lung, testes, lymphatic nodes, and especially spleen, were transduced. These findings indicate that intracarotid application of AV and lipoDNA vectors can effectively transduce tumor cells in the brain, and that BTB modulation by BK infusion can further increase the number of transgene-expressing tumor cells.

OVERVIEW SUMMARY

Selective intravascular delivery of transgenes to intracranial tumors in rat models can be effected by replication-defective adenovirus vectors and by liposome–DNA complexes bearing cytoplasmic expression cassettes. Delivery in both cases is enhanced by coinfusion of the pharmacologic agent bradykinin, which preferentially facilitates passage across the blood–tumor barrier, as compared to the blood–brain barrier. This delivery modality targeted both the main tumor mass and small tumor foci infiltrating the surrounding brain parenchyma. Liposome–DNA complexes proved even

[1]*Molecular Neurogenetics Unit, Neuroscience Center, Massachusetts General Hospital, and Neurology Department, Harvard Medical School,* Boston, MA 02129.
[2]Department of Neurosurgery, Faculty of Medicine, Martin-Luther-University Halle-Wittenberg, Halle, Germany.
[3]Neuro-Oncology Laboratory, Neurosurgical Service, Massachusetts General Hospital, Boston, MA 02114.
[4]Present address: Canji, Inc., Schering-Plough Company, San Diego, CA 92121.
[5]*Neurosurgical Service, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.*
[6]Present address: Division of Neurosurgery, Scripps Clinic, La Jolla, CA 92037.

more efficient than adenovirus vectors in this context, reflecting both efficient entry across the blood–tumor barrier and immediate transgene expression in the cell cytoplasm without need for transport to the nucleus. This method provides a means to seek out invasive tumor foci through intravascular injection of gene delivery vehicles that selectively pass into tumors through their newly forming vasculature.

INTRODUCTION

GENE THERAPY for brain tumors has entered the stage of clinical trials, but many problems remain. A major limitation is the inability to transduce a large enough pool of tumor cells to confer cytotoxicity to the whole tumor (Kramm *et al.*, 1995). In addition, the delivery must be tumor selective to reduce toxicity to normal brain. Finally, choice of vector affects both transduction efficiency and tumor selectivity, as well as transgene expression, replication within tumor, immunogenicity, and both central and peripheral toxicity (Chiocca *et al.*, 1994; Schofield and Cuskey, 1995).

Three modes of vector delivery to experimental brain tumors have been studied: stereotactic intratumoral inoculation (Boviatsis *et al.*, 1994a; Viola *et al.*, 1995), intrathecal application (Kramm *et al.*, 1996), and, most recently, intraarterial application (Neuwelt *et al.*, 1991; Doran *et al.*, 1995; Nilaver *et al.*, 1995; Rainov *et al.*, 1995). Stereotactic intratumoral application has the advantage of a high spatial accuracy with high local vector titers, but suffers from distribution being limited to a few millimeters surrounding the injection site, and depends on the biological properties of the vectors used (Boviatsis *et al.*, 1994b). Intrathecal delivery of vectors seems to be limited to treating intrathecal neoplasms or to attacking the intrathecal parts of intracerebral tumors (Viola *et al.*, 1995; Kramm *et al.*, 1996). Intraarterial vector application with or without disruption of the blood–brain barrier (BBB) or the blood–tumor barrier (BTB) offers a solution for the vector distribution difficulties by employing the well-developed tumor neovasculature for transgene delivery to all vascularized tumor foci. In contrast to the normal BBB, which limits the size of substances entering from the blood stream into brain cells and brain interstitial space, brain tumor neovasculature features a more permeable barrier (Cox *et al.*, 1976; Inamura and Black, 1994). However, the BTB still limits delivery of high molecular weight substances to tumor tissue and to immediately adjacent, partly tumor-infiltrated areas of the brain (Hoshino, 1984). The ability to achieve selective pharmacological opening of the BTB using bradykinin (BK) and its derivatives facilitates the selective delivery of cytotoxic drugs and virus vectors to intracerebral neoplasms (Rainov *et al.*, 1995; Elliot *et al.*, 1996; Barnett *et al.*, 1998), as the BTB is less rigid than the BBB.

Some of the virus vectors, such as retrovirus, integrate into the host genome and can cause insertional mutagenesis or oncogene activation, while others, such as herpes simplex virus type 1 (HSV), do not integrate, but can potentially cause encephalitis or other neurotoxicity. Liposome–DNA complexes (lipoDNA) represent alternative vehicles for gene transfer and avoid some of the unwanted features of virus vectors (Hug and Sleight, 1991). They have been shown to be highly effective in transfecting cultured cells, although different liposomal formulations have different transfection efficiencies and abilities to carry varying amounts of DNA (San *et al.*, 1993; Felgner *et al.*, 1994; Kramm *et al.*, 1995;/ Stephan *et al.*, 1996). LipoDNA complexes probably fuse with cell membranes, releasing DNA into the cytoplasm (Hug and Sleight, 1991), although the precise structures formed by lipids and DNA remain somewhat controversial (Felgner *et al.*, 1995). The route of administration may dramatically affect the uptake of liposomes by normal and tumor cells. *In vivo* studies showed that intravenously injected liposomes are taken up mainly by the reticuloendothelial system (RES), particularly in the liver and spleen (Hug and Sleight, 1991). Intraarterial application of lipoDNA in experimental animals is, however, still not well investigated, and little is known about transduction efficiency in brain tumors *in vivo* (Yoshida and Mizuno, 1994).

This study addresses the efficiency and selectivity of intraarterial delivery of replication-deficient AV vectors and lipoDNA with or without pharmacological modulation of the BTB.

MATERIALS AND METHODS

Virus vector

The virus vector used in this study was a recombinant adenovirus type 5 (Ad5) with disruption of the E1B region, with the *Escherichia coli lacZ* gene encoding β-galactosidase (β-Gal) placed under the control of the cytomegalovirus (CMV) promoter (kind gift of Genzyme, Cambridge, MA). Viral stocks were generated in 293 cells, concentrated by ultracentrifugation, titered by plaque assays, and stored at −80°C. Ten microliters of the virus stock (a total of 1×10^9 PFU) was dissolved in 1 ml of warm (37°C) 0.9% NaCl and immediately introduced into the carotid artery as a bolus injection.

Liposome–DNA complexes

The T7T7T7Bgal plasmid (Progenitor, Columbus, OH) was grown in *E. coli* HMS174 cells (Novagen, Madison, WI) and isolated by Maxi-prep (Qiagen, Santa Clarita, CA). Ten micrograms of DNA was incubated with 25 U of T7 RNA polymerase (New England BioLabs, Beverly, MA) and then mixed with 24 μg of LipofectAMINE (Life Technologies, Gaithersburg, MD) in 200 μl of Opti-MEM (Life Technologies) at room temperature. LipoDNA was injected immediately after preparation.

Animal studies

Twenty adult male Fischer 344 CD rats (Charles River Laboratories, Wilmington, MA) weighing 200–250 g were used in this study. There were four groups: BK infusion and adenovirus (AV) injection ($n = 5$), vehicle infusion and AV injection ($n = 5$), BK infusion and lipoDNA injection ($n = 5$), and vehicle infusion and lipoDNA injection ($n = 5$). Procedures and housing of animals were performed in accordance with the guidelines issued by the Massachusetts General Hospital Subcommittee on Animal Care. Intracerebral solitary 9L tumors were produced

by stereotactic inoculation of 1×10^5 tumor cells in 5 μl of Dulbecco's modified Eagle's medium (DMEM; Life Technologies) into the right frontal lobe of the rats. Anesthesia and implantation technique were as described elsewhere (Rainov *et al.*, 1995).

Seven days after tumor implantation, animals were reanesthetized for intracarotid catheterization. The right common carotid artery (CCA) was exposed through a 3-cm midline incision, as previously described (Rainov *et al.*, 1995). Bradykinin acetate (Sigma, St. Louis, MO) was dissolved in distilled water (25 μg/ml) and kept on ice. Immediately prior to infusion the solution was warmed to 37°C and infused at a rate of 10 μg/kg/min for 10 min using a microsyringe pump (Medfusion Systems, Norcross, GA). BK infusion was immediately followed by injection of virus or lipoDNA in 1 ml of vehicle over 5 min.

Fixation, sectioning, and staining

For histochemical analysis of marker gene expression, rats were sacrificed 48 hr after intracarotid infusion by a lethal dose of sodium pentobarbital (15 mg) and transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Brain, spinal cord, and internal organs—liver, lung, kidney, heart, testes, and lymph nodes—were placed in 30% sucrose for cryoprotection, frozen 2–4 days later over liquid nitrogen, and stored at −80°C until further processing (not longer than 6 weeks). Tissue blocks were mounted in O.C.T. compound (Miles, Elkhart, IN) and 20-μm-thick serial sections were cut on a Jung cryostat (Cryocut 2800 E; Leica, Deerfield, IL). Every fifth section in the tumor area and every tenth section in normal brain, cerebellum, and internal organs was mounted on silane-coated slides (Superfrost, Fisher, Pittsburgh, PA) and air dried at room temperature. Tissue sections were stained for *lacZ* expression by histochemistry using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate (Sigma), as previously described (Turner *et al.*, 1990). After rinsing with water for 5 min, sections were counterstained with Meyer's hematoxylin (Sigma) or neutral red (Fisher), dehydrated in ethanol, immersed for 5 min in xylene, and then mounted under coverslips. Control sections of animals not treated in the present study were included to assure specificity of the staining process.

Cell counting and evaluation of sections

LacZ-positive and -negative cells were counted at a 200-fold magnification using a light microscope (Nikon, Garden City, NY). Evaluation of sections was carried out in a blinded fashion. For the study of lipoDNA gene transfer, 1000 cells were counted in each of three random visual fields throughout the tumor, since the pattern of distribution was fairly homogeneous. In animals with AV injection, small foci (<0.5 mm) were evaluated by counting total numbers of stained and nonstained cells. In larger tumors (2–5 mm), three random visual fields in the tumor periphery (defined as the part of tumor remaining in the microscopic visual field when normal brain tissue is also visible) and three random fields in the tumor center were counted and values averaged. Normal brain and internal organs were evaluated qualitatively.

Statistical evaluation

For calculation of mean values and standard error of the mean, as well as for all significance tests, such as *F* test and Student's *t* test, the SPSS for Windows software package was used.

RESULTS

AV application

When 1×10^9 PFU of AV was injected into the internal carotid artery in the absence of BK, 3–9% (mean, 6.2%) of tumor cells in large tumor foci became positive for β-galactosidase (β-Gal) (Fig. 1). In small foci, a higher proportion of cells showed transgene expression (mean, 14.4%; range, 10–25%). The tumor periphery, especially when in contact with cerebrospinal fluid (CSF), appeared to have a higher percentage of transgene-expressing cells as compared with the tumor center. After BK infusion, the number of positive cells in large tumors increased to 17.5% (range, 12–32%), while the increase in transduction in small tumor foci was less pronounced (mean, 25.1%; range, 16–37%) and without a distinctive pattern of distribution (Fig. 2).

Some endothelial cells in normal brain capillaries expressed β-Gal with and without BK. No increase in the number of these cells was seen with BK. There was no intrinsic β-Gal positivity of capillaries in control brain sections without vector application (control tissues from a parallel study were stained and evaluated; Barnett *et al.*, 1998). Of all peripheral organs examined, the liver showed the highest density of β-Gal-expressing cells, followed by the lung (data not shown). There was no histochemical positivity for the transgene in the heart, spleen, and testis. Strong β-Gal positivity of kidney and choroid plexus was seen in all sections and considered to be due to high constitutive expression of mammalian β-galactosidase or low endogenous pH of tissue.

LipoDNA application

LipoDNA was diluted in 0.9% NaCl to a total volume of 1 ml (immediately before injection). In the absence of BK, 22–47% (mean, 39.8%) of tumor cells were transduced. There was no difference in transduction efficiency in small and large tumor foci and no marked ring pattern of transgene distribution, such as in the case of AV (Fig. 3). BK infusion further increased the amount of β-Gal-expressing tumor cells (mean, 53%; range, 33–71%).

In normal brain, many endothelial cells in capillaries and apparently normal glial cells expressed the marker gene. BK did not appear to influence these numbers. Organs of the reticuloendothelial system (RES), such as spleen and lymph nodes, showed frequent expression of the marker gene.

DISCUSSION

This series of experiments was carried out to investigate the transduction efficiency of intracarotid replication-deficient AV

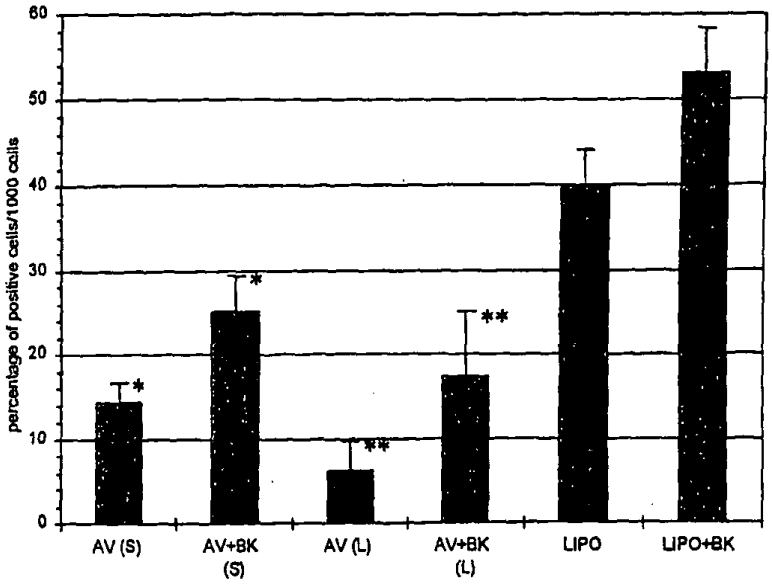

FIG. 1. Graph showing percentage of β-Gal-expressing cells/1000 cells in tumors. Data are expressed as mean and standard error of the mean (SEM, bars). Asterisks indicate statistically significant differences between the values ($^*p < 0.05$, $^{**}p < 0.01$).

vectors and liposome–DNA complexes in the presence and absence of the BTB modulator, BK, in a rat 9L gliosarcoma model. Without BK, AV injection rendered a mean percentage of 6.2% tumor cells positive for β-Gal in large tumors and 14.4% in small tumor foci (<0.5 mm). BK increased the number of X-Gal-positive cells in large tumors 2.8-fold, and 1.7-fold in small tumor foci. With AV application, only a few capillaries in the normal brain stained positive for β-Gal. With lipoDNA injection, transduction occurred to a similar extent (39.8%) in both large and small tumor foci. Marker gene expression in tumor cells increased 1.3-fold in the presence of BK. With lipoDNA, LacZ-positive endothelial cells and glia were found in normal brain tissue. Both types of vectors produced marker gene expression in peripheral organs, but AV showed greater transduction of liver cells, while lipoDNA showed preferential labeling of the RES.

AV vectors

The extent of transduction of the target cells is one of the key factors that determines the efficacy of AV-mediated gene therapy for neoplastic diseases (Hunrd *et al.*, 1995). Different routes of AV application result in different efficiencies of gene transfer. Direct intratumoral injection has been the most often used and best established application mode for all viral vectors (Boviatsis *et al.*, 1994a; Chen *et al.*, 1994; Badie *et al.*, 1995; Maron *et al.*, 1996; Vincent *et al.*, 1996). Intratumoral injections of AV (1×10^7 to 1×10^9 PFU) have been used mostly

FIG. 2. (A) Photomicrograph of a small tumor focus in the AV group 48 hr after virus injection in the absence of BK. Note the high number of stained cells throughout the tumor (original magnification, ×200; 20-μm frozen section; X-Gal staining). (B) Photomicrograph of a small tumor focus in the AV group 48 hr after virus injection and BK infusion. Increased number of stained cells throughout the tumor (original magnification, ×200; 20-μm frozen section; X-Gal staining). (C) Photomicrograph of a large 9L gliosarcoma in the BK-AV group 48 hr after virus injection in the absence of BK (original magnification, ×100; 20-μm frozen section). (D) Large 9L tumor in the BK-AV group 48 hr after virus injection and BK infusion (original magnification, ×100; 20-μm frozen section). All sections were counterstained with neutral red.

FIG. 3. (A) Photomicrograph of a tumor in the lipoDNA group 48 hr after injection in the absence of BK. Note the high number of stained cells throughout the tumor (original magnification, ×100; 20-μm frozen section; X-Gal staining). (B) Photomicrograph of a tumor in the BK-lipoDNA group 48 hr after virus injection and BK infusion. Increased number of stained cells throughout the tumor (original magnification ×200; 20-μm frozen section, X-Gal staining). (C) Photomicrograph of large 9L tumor and surrounding normal brain in the BK-lipoDNA group 48 hr after virus injection and BK infusion (original magnification, ×200; 20-μm frozen section). Note positivity of peripheral capillaries sprouting towards the tumor border (arrows). (D) Photomicrograph of normal brain in the lipoDNA group 48 hr after virus injection in the absence of BK (original magnification, ×200; 20-μm frozen section). Note the stained endothelial cells (blue) along normal brain capillaries (arrows). All sections were counterstained with hematoxylin and eosin (H&E).

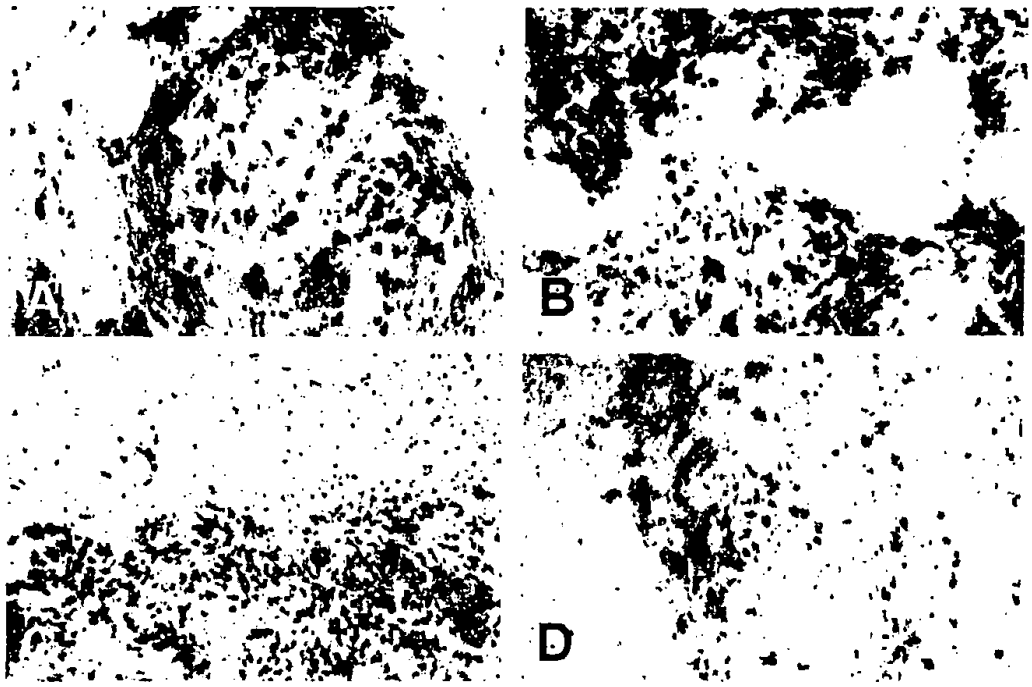

FIG. 2.

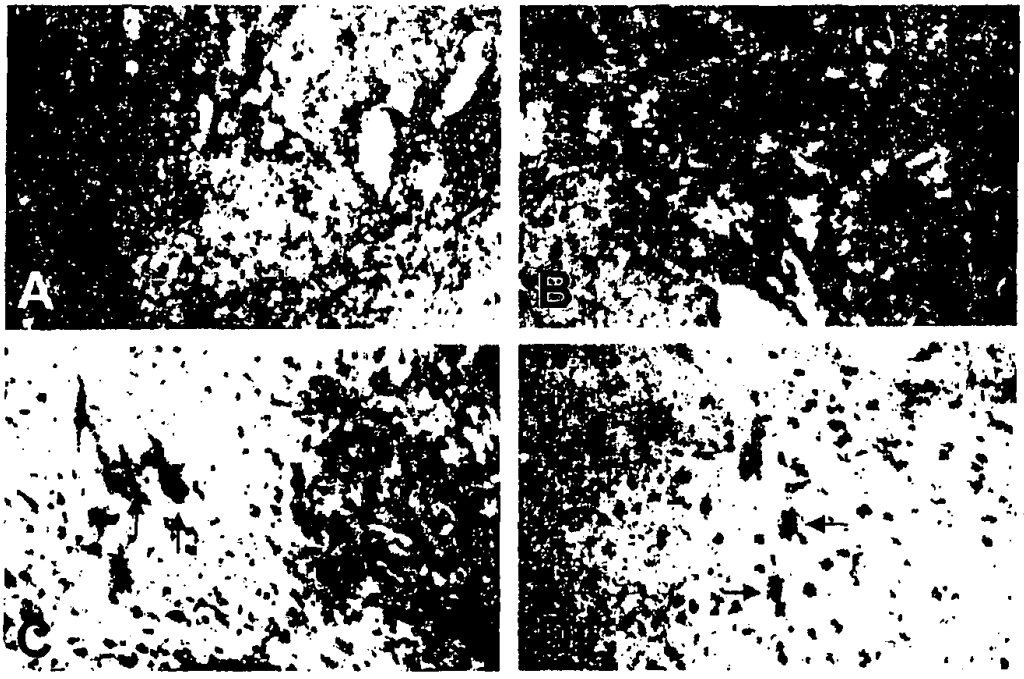

FIG. 3.

as a single dose in a few microliters of vehicle (Perez-Cruet *et al.*, 1994; Badie *et al.*, 1995). This amount is comparable with the virus dose used in the present study (1×10^9 PFU); however, unlike intraarterial application, direct intratumoral injection produces high local transduction rates, but is unable to access the peripheral parts of the tumor.

Viola *et al.* (1995) injected AV vectors intratumorally and into the CSF space, either simultaneously with intrathecal 9L tumor inoculation or after establishing leptomeningeal cancer. AV injection into normal brain resulted in diffuse transduction of normal cells at the injection site, while intratumoral injection of AV vector into cerebral gliomas resulted in efficient tumor cell transduction (Viola *et al.*, 1995). Intrathecal AV injection in rats with meningeal tumor spread produced a relatively high transduction of the infiltrating tumor in the subarachnoid space when virus was given simultaneously with tumor inoculation. Transduction rates of both solid and leptomeningeal tumors correlated with the number of injected particles. Using a C6 glioma model, Chen *et al.* (1994) injected 3×10^8 AV vectors bearing the herpes simplex virus thymidine kinase gene (HSV *tk*) into established tumors and subsequently treated the animals with ganciclovir (GCV), resulting in a 500-fold reduction in tumor volume, as compared with controls.

As intratumoral application of AV vectors is entering the stage of clinical trials (Eck *et al.*, 1996), its efficiency needs to be improved considerably. Human tumors have a different morphology (Berens *et al.*, 1990) and a larger volume than in animal models, and so intratumoral injections alone seem insufficient to transduce high numbers of tumor cells. Combined approaches such as simultaneous intratumoral and intraarterial injections may yield the highest gene transfer rates, as intratumoral injection may achieve a sufficient local vector concentration and intraarterial delivery may preferentially access the periphery of tumor as well as small tumor foci infiltrating the adjacent brain. Our results demonstrate that intraarterial AV vectors are capable of a highly efficient transduction of small tumor foci, possibly owing to their active neoangiogenesis (Cox *et al.*, 1976; Berens *et al.*, 1990).

AVs are stable particles with a diameter of 70–100 nm, which can be produced in high titers, e.g., 1×10^{12}/ml. Drawbacks of AV vectors are their high immunogenicity and the preexisting immunity against AV in humans, which is responsible for rapid elimination of vectors (Eck *et al.*, 1996). In cancer therapy, however, the application of a high single dose of AV vectors may be able to overcome virus inactivation by the immune system, at least for a limited time. On the other hand, systemic immune response against AV vectors may be beneficial for limitation of spread to peripheral organs. In our experiments in rats a relatively high level of transduced cells was noted in peripheral organs after a single intracarotid injection of 1×10^9 PFU of AV, this apparent obstacle may be overcome in humans by the anti-AV immune response.

AV vectors are highly efficient in transducing glioma cells *in vivo* when compared with herpes simplex virus type 1 (HSV) vectors. In a previous study with intraarterial delivery of HSV vectors (Rainov *et al.*, 1995), less than 5% of tumor cells in the periphery, and less than 2% in the tumor center, of intracerebral 9L tumors were transduced in the absence of BK. In the analogous experimental setting of the present study, 6 to 14% of the tumor cells, mostly in the tumor periphery, were transduced by intracarotid AV vector application without BK modulation of the BTB. The differences in transduction efficiency may reflect the biological or physical differences of the vectors (for example, AV particles are about 85 nm in diameter while HSV is about 150 nm) and may vary with different types of tumors.

LipoDNA delivery

Synthetic vectors such as liposomes represent an arguably safer approach to gene therapy for brain tumors. They are generally easy to produce in large quantities, and they do not carry the risks implicit in viruses (Kramm *et al.*, 1995). On the other hand, design strategies have not been optimized to facilitate high efficiency cell penetration, to avoid degradation by cellular endonucleases, and to optimize transport to the nucleus.

Many studies have investigated the effects of various lipids (Behr *et al.*, 1989), lipid/DNA ratios (San *et al.*, 1993), DNA condensation (Curiel, 1994; Fritz *et al.*, 1996), and addition of targeting (Michael and Curiel, 1994) of fusogenic agents (Tomita *et al.*, 1995) on liposome-mediated gene transfer in cultured cells. However, *in vivo* liposome-mediated gene transfer is still in the preliminary stages. Zhu *et al.* (1996) and Zerrouqi *et al.*, (1996) studied direct infusion of liposomes complexed with an HSV *tk*-expressing plasmid into tumors using an osmotic minipump delivery and effected marked tumor regression in GCV-treated animals.

To expedite high and rapid transgene expression, the plasmid T7T7T7Bgal (Progenitor) was used. This plasmid, when primed with a small amount of T7 RNA polymerase, generates more RNA polymerase and large levels of transgene products directly in the cell cytoplasm (Chen *et al.*, 1995). When complexed with Lipofectin this plasmid transduced more than 30% of cultured cells, and when injected directly into tumors resulted in transgene expression in a limited area surrounding the injection site (Chen *et al.*, 1996). Our results indicate that an intraarterial approach using lipoDNA can yield highly efficient and more extensive gene transfer to tumors *in vivo*, with the addition of BTB modification with BK improving the overall efficiency of delivery even further.

Role of BTB modulation by BK

Several mechanisms could account for increased permeability of the BTB after pharmacological disruption. These include increased vesicular transport (pinocytosis/endocytosis) through the endothelial cells and opening of the tight junctions between these cells (Elliot *et al.*, 1996; Inamura and Black, 1994). Bradykinin seems to affect predominantly the tight junctions by a receptor-mediated action on the cytoskeleton (Hess *et al.*, 1992). We (Rainov *et al.*, 1995) and others (Inamura and Black, 1984; Elliot *et al.*, 1996) have demonstrated selective permeabilization of the BTB for low and high molecular weight substances, and for viral and nonviral particles. It is also possible that BK exerts some effects on the metabolism of glioma cells and thus increases their transducibility, similarly to the increase in transducibility seen with other viral vectors after irradiation of the target cells (Advani *et al.*, 1998).

In conclusion, this study demonstrates that viral and nonviral vectors can be effectively targeted to experimental brain tu-

mors by intracarotid application with or without pharmacological modulation of the BTB

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Address reprint requests to:
Dr. Xandra O. Breakefield
Massachusetts General Hospital East
Department of Molecular Neurogenetics
13th St., Bldg. 149, 6th floor
Charlestown, MA 02129

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Pre-existing herpes simplex virus 1 (HSV-1) immunity decreases, but does not abolish, gene transfer to experimental brain tumors by a HSV-1 vector.

Herrlinger U, Kramm CM, Aboody-Guterman KS, Silver JS, Ikeda K, Johnston KM, Pechan PA, Barth RF, Finkelstein D, Chiocca EA, Louis DN, Breakefield XO.

Neurology, MGH Cancer Center, Massachusetts General Hospital, Charlestown 02129, USA.

The influence of pre-existing anti-herpes simplex type 1 (HSV-1) immunity on HSV-1 vector-mediated gene transfer to glioma cells was analyzed in this gene marking study using intracranial D74 gliomas in syngeneic Fischer rats. The HSV-1 mutant virus used, hrR3, is defective in ribonucleotide reductase and bears the marker genes E. coli lacZ and HSV-1 thymidine kinase (HSVtk). Initial marker gene expression in tumors 12 h after direct virus injection was reduced in immunized animals to about 15% of that in nonimmunized animals. Marker gene expression in both sets stayed at initial levels for 2 days after intratumoral injection and declined markedly on day 5. Inflammatory infiltrates in the tumor were more prominent in HSV-1-immunized, as compared with nonimmunized animals, at 12 and 24 h, but appeared similar at 2-5 days after injection. By day 10, the immune reaction had subsided in immunized animals and macrophages remained only in nonimmunized animals. In conclusion, gene transfer to brain tumors using a HSV-1 vector was greatly reduced, but not completely abolished, under pre-immunization conditions. Pre-existing antibodies to HSV-1 may also serve a positive role in providing an increased margin of safety in intracranial application of HSV-1 vectors by limiting spread of the virus within the brain and to other tissues.

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Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses

KEIRO IKEDA[1], TOMOTSUGU ICHIKAWA[1], HIROAKI WAKIMOTO[1], JONATHAN S. SILVER[2], THOMAS S. DEISBOECK[1], DIANNE FINKELSTEIN[3], GRIFFITH R. HARSH IV[1], DAVID N. LOUIS[1,2], RAYMOND T. BARTUS[4], FRED H. HOCHBERG[5] & E. ANTONIO CHIOCCA[1]

Molecular Neuro-Oncology Laboratories, [1]Neurosurgery Service, [2]Neuropathology, [3]MGH Cancer Center and [5]Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129, USA
[4]Alkermes, Cambridge, Massachusetts 02111, USA
Correspondence should be addressed to E.A.C.; email: Chiocca@helix.mgh.harvard.edu

The occurrence of multiple tumors in an organ heralds a rapidly fatal course. Although intravascular administration may deliver oncolytic viruses/vectors to each of these tumors, its efficiency is impeded by an antiviral activity present in complement-depleted plasma of rodents and humans. Here, this activity was shown to interact with complement in a calcium-dependent fashion, and antibody neutralization studies indicated preimmune IgM has a contributing role. Short-term exposure to cyclophosphamide (CPA) partially suppressed this activity in rodents and humans. At longer time points, cyclophosphamide also abrogated neutralizing antibody responses. Cyclophosphamide treatment of rats with large single or multiple intracerebral tumors substantially increased viral survival and propagation, leading to neoplastic regression.

The treatment of tumors that have seeded multiple sites in an organ, such as brain or liver, remains palliative. Gene therapy vectors delivering new anticancer treatments such as wild-type p53 or chemotherapy-sensitizing or immune-enhancing cDNAs are being delivered through direct injection into tumors[1–7]. In some cases, evidence for local regression has been found. However, local anticancer action against one tumor mass may not necessarily provide an anti-neoplastic effect against other tumor masses in the organ.

Oncolytic viruses have been used as anti-tumor agents both experimentally and clinically[2,4–14]. They also can function as gene therapy vectors by delivering anticancer genes. Transduction of syngeneic rat 9L tumors, established in the brains of immunocompetent rats, by intra-arterial herpes simplex virus (HSV) type 1 vector occurred after pharmacologic disruption of the blood–tumor–brain barrier[15,16]. However, tumor infection remains relatively inefficient, because a substantial antiviral response eliminates the viral vector. Here, we show this response is innate and differs from elicited neutralizing humoral responses or complement, although it interacts with the latter. Antibody neutralization studies show that preimmune IgM contributes to this activity. Concomitant treatment with cyclophosphamide (CPA) partially suppressed this innate antiviral response and decreased plasma levels of total IgM. At longer time points (more than 4 days), CPA also suppressed neutralizing antibody responses. The addition of CPA to an intravascular viral vector and a blood–brain barrier modifier resulted in a substantial increase both in the number of rats with transduced tumors and in the anatomic extent of tumor transduction. This can lead to tumor regression and long-term survival even in animals with multiple intracerebral tumors.

Generation of viral neutralizing antibodies

We sought to characterize elicited immune responses against an intravascular oncolytic HSV type 1 virus (hrR3). Immunocompetent Fischer 344 rats exposed to intracerebral hrR3 form neutralizing antibodies[17]. As expected, in both immunocompetent and athymic rats a neutralizing antibody response against virus was elicited 4 days after intra-arterial injection of hrR3, and was abrogated by simultaneous treatment with CPA (Fig. 1*a* and *b*).

Presence of a CPA-labile, innate antiviral activity in rat plasma

Intra-arterial administration of virus should result in initial infection of some cells in the tumor, with initial generation of progeny viruses (12–18 hours) and subsequent rounds of infection and propagation (24–48 hours). The above results show that neutralizing antibody responses, detected at 4 days, are not likely to affect these early processes. However, pre-incubation of hrR3 with complement-depleted, undiluted plasma, prepared from preimmune immunocompetent rats (Fig. 2*a*) or athymic rats (Fig. 2*b*) substantially reduced *in vitro* viral transduction. These results indicate that an innate antiviral activity is present in rat plasma. CPA suppressed the antiviral effect of undiluted, immunocompetent rat plasma by approximately 30% (Fig. 2*a*) and that of athymic rat plasma (1:2 dilution) by approximately 20% (Fig. 2*b*). These results indicate that animal exposure to CPA leads to a partial suppression of this innate antiviral activity.

To determine if this antiviral activity required initial contact with virus or directly protected cells from viral infection, we added plasma onto monkey kidney (Vero) cells in culture before assaying for hrR3 activity. There was no inhibition of viral transduction, indicating that this activity acted directly on virus (Fig. 2*c* and *d*).

If the activity was not innate but was an early elicited response undetected by the serological experiments in Fig. 1, pre-exposure of rats to virus for 2 days (that is, before the detectable rise in neutralizing antibody titers) would be expected to augment plasma's ability to inactivate virus. However, there was no difference in the antiviral activity of plasma prepared from preimmune rats or from rats exposed to virus for 2 days (Fig. 2*e* and *f*). There also

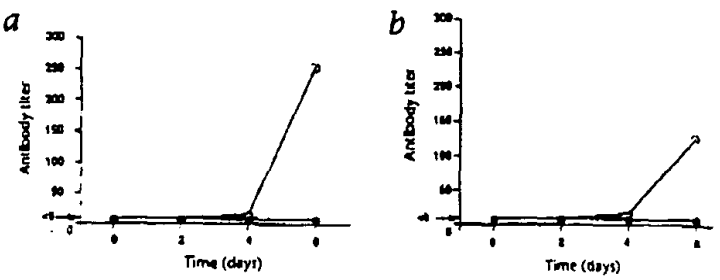


Fig. 1 CPA inhibits the generation of neutralizing antibodies in immunocompetent or athymic rats. Plasma was prepared from Fischer 344 CD rats (*a*) or nude rats (*b*) on days 0, 2, 4 and 8 after injection with hrR3, RMP-7 and saline (○) or CPA (●), and titers of neutralizing antibodies against HSV were assayed. Arrows, titers below 8.

was no difference in the extent of suppression of this activity by CPA. This indicates that short-term (that is, before the formation of neutralizing antibodies) viral exposure does not change the magnitude of the innate activity. These results indicate that there was an innate antiviral activity in rat plasma that was different from complement and was partially suppressed by CPA (Fig. 2*a*,*b*,*e* and *f*), and had to interact with virus before protecting cells from infection (Fig. 2*c* and *d*).

The CPA-labile, innate antiviral activity is present in humans

The above experiments could be explained if a natural antibody against HSV (a human virus) was present in rat plasma. We collected plasma from 3 of 20 human patients, who did not have pre-existing antibodies against HSV, before and after they underwent chemotherapy with CPA. This plasma, after complement depletion, still had antiviral activity, which was suppressed by CPA treatment (Fig. 3*a*). The innate antiviral action was even stronger, but was still partially suppressed by CPA (Fig. 3*b*) when complement was not inactivated. This result indicates that humans also have innate antiviral function(s) that can be suppressed by CPA. To further test the 'natural antibody' hypothesis, we grew hrR3 in rat cells, to allow the virus to acquire an envelope from rat membranes with its antigenic characteristics[16]. However, there was no difference in the antiviral activity of rat plasma against hrR3 grown in rat, human or Vero (monkey) cells (data not shown), again indicating that a 'natural antibody' t ory did not provide a suitable explanation for this activity.

The innate antiviral activity interacts with complement

Although the total plasma level of complement was not affec by CPA (data not shown), we sought to determine if its funct was. Serially diluted and unheated ('complement-rich') plas from immunocompetent or athymic rats (Fig. 4*a* and *b*) was m active in inhibiting virus than was complement-depleted, hea plasma (Fig. 2*a* and *b*). Plasma from immunocompetent rats in tivated more than 99% of the virus at a dilution of 1:16 (Fig. 4 CPA suppressed this by approximately 10%. Plasma from athy rats inactivated approximately 80% of the virus at a dilution 1:32. CPA suppressed this by approximately 60% (Fig. 4*b*). Co venom factor inhibits complement by depleting C3 and l components[19]. The antiviral activity of unheated plasma fr rats treated with cobra venom factor was also inhibited (Fig and *b*). Treatment with EGTA-Mg depletes Ca^{2+}, an ion neede activate complement through the 'classical pathway' (ref. 2 'lectin pathway' (ref. 21). Pretreatment of unheated plasma v EGTA-Mg was also suppressive (Fig. 4*a* and *b*). These results ir cate that the innate antiviral activity interacted with complem in a calcium-dependent manner and that the suppression of activity by CPA altered complement's antiviral function.

Further characterization of the innate antiviral activity

Complement activation can occur by interaction with munoglobulin ('classical pathway') or lectin ('lectin pathway') calcium-dependent manner or spontaneously ('alternative p way') in a calcium-independent manner. The above results vide evidence for involvement of the 'classical pathway' or 'le pathway'. Preliminary experiments seemed to exclude the p bility of interaction with lectin (K.I and E.A.C., data not sho However, pretreatment of plasma with rabbit IgG raised against IgM reversed the innate antiviral activity of plasma in a dose pendent manner, whereas treatment with control rabbit pre mune IgG did not (Fig. 5*a*). This indicated that preimmune contributed to the innate antiviral activity of plasma. We also

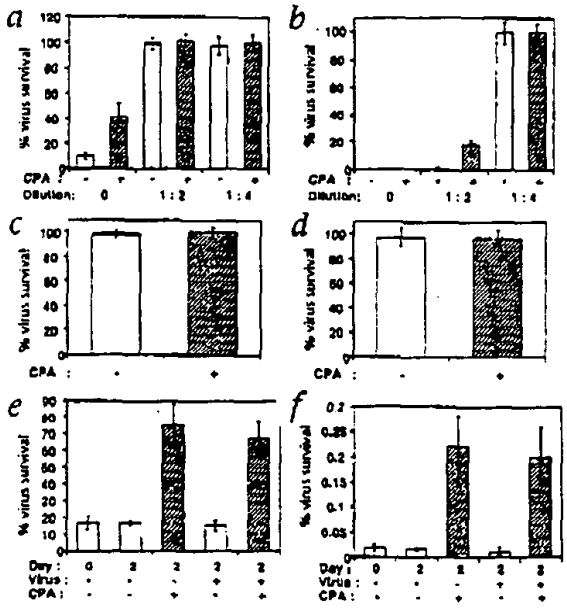
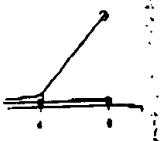


Fig. 2 Presence of an innate antiviral activity in rat plasma that is CP bile. Plasma was prepared from immunocompetent rats (*a*, *c* and *e*) athymic rats (*b*, *d* and *f*) 2 d after intraperitoneal CPA (or carrier) admini tion. *a* and *b*, Serial dilutions of heated plasma were used in the inactiv assay. *c* and *d*, Heated and undiluted plasma was added onto Vero ce culture and washed off the cells before hrR3 activity assay. *e* and *f*, Flu was prepared before (day 0) or 2 days after intravascular administratio hrR3 (or mock injection). For *f*, undiluted athymic rat plasma was used, low values (compared with those of *e*) reflect the more potent antiviral a ity of undiluted athymic rat plasma, and correspond to the almost u tectable levels of the first two bars in *b*.



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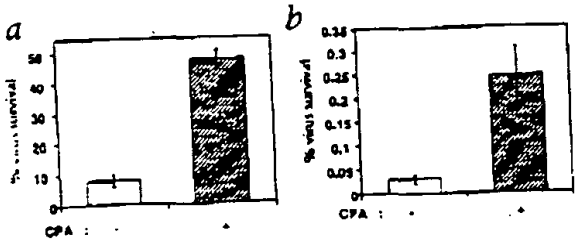


Fig. 3 The innate antiviral activity is also present in human plasma. Human plasma was collected from patients before (–) and after (+) they underwent chemotherapy with CPA. Heat-treated (*a*) and unheated (*b*) plasma was used in the inactivation assay.

pared plasma from CPA-treated (or control) rats and then pre-incubated it with rabbit antibody against rat IgM. The CPA and antibody treatments generated an even more profound reversion of innate antiviral activity (Fig. 5*b*). At this dilution of plasma (1:8), there was little or no effect from CPA treatment (Fig. 4*b*), but antibody neutralization of rat IgM resulted in further increase in virus survival after treatment with CPA (Fig. 5*b*). These results indicate that CPA decreased the concentration of rat preimmune IgM available to interact with the oncolytic virus (Fig. 5*b*).

Further support for the involvement of CPA in reversing the role of preimmune IgM in the suppression of viral activity was provided by assays of immunoglobulin plasma levels in rats before and after treatment with CPA. The average level of IgM in plasma was 50.4 mg/dl before CPA ($n = 4$), but had decreased considerably, to 19.6 mg/dl, 2 days after CPA treatment. There was no change in complement levels. With the previous results, these data indicate that preimmune IgM contributed substantially to the observed innate antiviral activity.

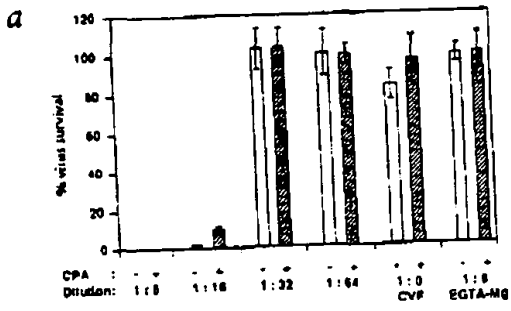
Given these results, we next asked if CPA increased the survival of oncolytic virus in intracerebral human glioma xenografts, directly injected with hrR3. We explanted tumors 2 and 4 days after direct stereotactic injection of hrR3 with or without concomitant administration of CPA, and measured yields of surviving virus. By day 2, there were approximately 1×10^6 plaque-forming units (PFU) of hrR3 in tumors from rats ($n = 3$) treated with CPA, whereas there were only 1×10^5 PFU of hrR3 in tumors from rats ($n = 3$) that had not been treated with CPA. By 4 days, there were still 1×10^6 PFU of hrR3 in tumors of CPA-treated rats, but only 1×10^3 PFU in contr... rats. These results thus directly correlate the *in vitro* evidence sho... ing an effect of CPA on increasing virus survival on exposure ... plasma with *in vivo* measurements showing an effect of CPA ... augmenting the survival of oncolytic virus in injected tumors.

CPA augments tumor infection by intravascular virus

After administration of the oncolytic virus hrR3 through ... carotid intra-arterial route, pharmacologic modification of t... blood–tumor–brain barrier allows for apparent transduction ... some tumor cells[12,19]. Thus, we next determined whether the ... dition of CPA resulted in an increase in the efficiency of tun... transduction by intravascular virus. In immunocompetent r... with syngeneic intracerebral rat 9L gliosarcoma tumors, C... provided a substantial increase both in the number of positiv... transduced tumors and in the area of tumor transduction, a... intra-arterial treatment of immunocompetent rats with hr... and RMP-7, a pharmacologic modifier of the blood–tumor–br... barrier[22–25](data not shown). Because rat 9L tumors do not s... port viral replication as efficiently as human tumors[2], we ... peated the same experiment in athymic rats with a single hun... glioma xenograft in their frontal lobes. As in immunocompet... rats, CPA provided a substantial increase both in the numbe... positively transduced tumors and in the area of tumor transd... tion by intra-arterial hrR3 and RMP-7 (Table 1). Tumors in r... rats treated with hrR3, CPA and RMP-7 had many lacZ-posi... and HSV antigen-positive plaques (Fig. 6*a* and Table ... However, only one of six rats treated with hrR3 and RM... showed evidence of large intratumoral plaques, and one of ... rats treated with hrR3 and CPA had a small intratumoral pla... (Fig. 6*a* and Table 1). In survival studies, more than 50% of ... athymic rats with a single, large intracerebral human gli... were cured by the combination of hrR3, CPA and RMP-7 ... 6*b*). Analysis of other organs (liver, spleen, lungs, heart) did ... show evidence of lacZ-transducing, replicating virus eith... day 2 or at death (data not shown). These studies demons... that CPA substantially increased both the anatomic extent o... tratumoral 'plaque' formation and the number of tumors ... tively infected by intra-arterial hrR3.

CPA allows for delivery of virus into multiple tumors

The most malignant and untreatable forms of intracerebral



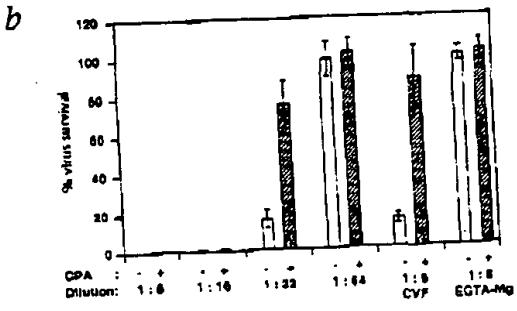


Fig. 4 The innate antiviral activity interacts with complement in a calcium-... dent manner. Plasma was prepared from immunocompetent rats (*a*) ... athymic rats (*b*) that had been injected intraperitoneally with CPA (+) or ... carrier (–) on day –2 and/or with cobra venom factor (CVF) or carrier on day ... –1 at a dose of 60 U/kg and on day 0 at 20 U/kg (days numbered with respect ... to the day rats were killed). Serial dilutions (below graphs) of unheated plasma were used in the inactivation assay. Plasma was also treate... EGTA-Mg before the assay. At a 1:8 dilution of plasma, innate antiviral ... abrogates viral survival, with very little effect from CPA. However, pre... ment with CVF or EGTA-Mg reverses partially (for CVF) or complete... EGTA-Mg) the antiviral effect of unheated plasma diluted 1:8. The add... CPA results in an even greater reversal in athymic rats (*b*).

plasms (malignant gliomas and metastatic tumors) manifest as multifocal and diffuse masses, located throughout the brain. To model such illnesses, we implanted human U87dEGFR glioma cells[25] into three separate intracerebral locations. These tumors grew rapidly and reliably, causing rat deaths by day 14 after implantation (results not shown). At day 8 after implantation, when tumors achieved a relatively large volume, producing clinical signs of illness, we treated rats with CPA (or vehicle) and intravascular hrR3 and RMP-7. We then collected brains 2, 4 and 8 days later to analyze the extent of tumor transduction. In some animals, by day 2, all three tumors showed evidence of small plaques; by day 4, more than 90% of tumor cells in all three foci were positively transduced; and by day 8, extensive involution and regression of the intracerebral masses was evident (Fig. 7*a*). We did a computer-assisted analysis of percent tumor transduction on days 2 and 4 after treatment in experimental rats (Table 2). Multiple tumor foci in a rat brain could be effectively transduced by intravascular administration of an oncolytic virus (Table 2). In survival studies, there was a statistically significant prolongation in the survival of rats with three separate intracerebral tumors after treatment with hrR3, RMP-7 and CPA (Fig. 7*b*), compared with survival after treatment with hrR3 and RMP7 ($P < 0.002$). Rats treated with mock injection and/or CPA perished by day 14 (data not shown). Rats that perished after treatment with hrR3, RMP7 and CPA showed evidence of lack of transduction of one or two tumors, whereas the other tumor(s) were infected and had involuted or were regressing (Fig. 7*c*). Analysis of other organs (liver, spleen, lungs, heart) did not show evidence of replicating virus either at the two-day time point or at death (data not shown). These findings thus provide evidence for the efficacy of this treatment against multiple intracerebral neoplasms.

Table 1 *lacZ* cDNA transduction and viral plaque formation in intracerebral human glioma xenografts, 2 d after intravascular delivery of oncolytic virus.

CPA	RMP7	Animals with intraneoplastic 'plaques'	% Intraneoplastic *lacZ* cDNA transduction in positive tumors[a]
–	–	0/4[b]	
–	+	1/6	73.7 (4.6)[c]
+	–	1/4	2.8 (0.2)
+	+	6/8	10.2 (3.6)
			51.3 (8.9)
			33.9 (9.2)
			2.7 (0.4)
			25.6 (7.5)
			18.8 (6.5)

[a]Percentages were calculated by computer-assisted analysis of *lacZ*-expressing tumor area divided by total tumor area. [b]Ratios represent the number of animals with visible, *lacZ*-expressing intraneoplastic 'plaques' divided by the number of treated animals. [c]Values represent the mean, with s.e.m. in parentheses.

Discussion

The exploration of host immune interactions with oncolytic virus is not only biologically important but also relevant for its therapeutic implications. Successful delivery of viruses and vectors through the vasculature may provide a means to target multiple tumors in an organ, a feat that has not been achieved so far in an efficient manner. Here we discovered that this inefficiency is related to inactivation of the vector in rat plasma and tumor. This inactivation could be partially circumvented by a single dose of cyclophosphamide. Addition of this agent substantially increased virus survival in the tumor, thereby increasing the number of rats whose tumors were positively infected and the anatomic extent of tumor transduction. This led to a significant prolongation of rat survival, by involution of the brain tumors. This was achieved in rats that had three separate intracerebral tumors, were treated when their illness was clinically detectable (on day 8 of a 14-day life span from tumor cell implantation) and showed radiologic evidence of intracerebral shift and edema (K.I. and E.A.C, results not shown).

Enduring, protective, T cell-independent IgM responses can be generated against some viruses[27] and our results indicate that such a response is elicited against HSV. The B-cell immunosuppressive agent cyclophosphamide (CPA) abrogated this response, which was detectable 4 days after virus administration. However, this time period fails to explain the considerable differences in the extent and number of transduced tumors 2 days after intra-arterial viral administration in the presence of CPA. An innate antiviral activity was thus discovered in plasma, whose function was partially suppressed by CPA. We did several experiments to characterize it. Clues to its identity were provided by experiments showing that rat preimmune IgM plasma levels decreased by more than half 2 days after treatment of rats with CPA (through its effects on B-cell production of immunoglobulin); plasma treatment with antibody against IgM removed this innate activity in a dose-dependent

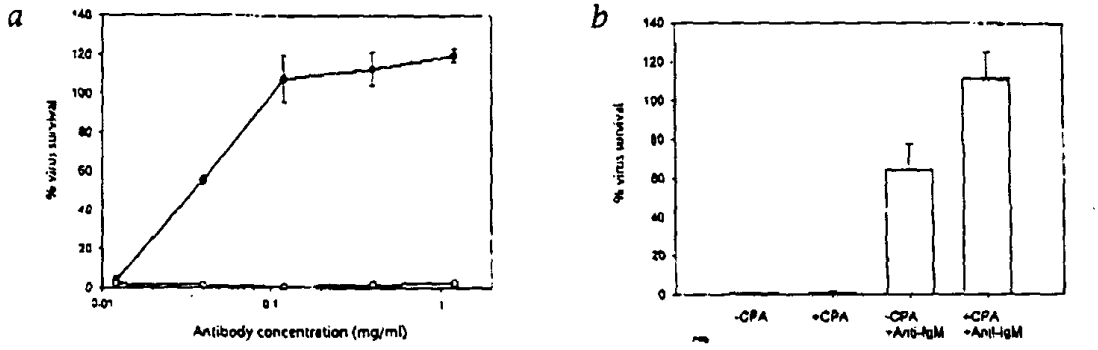


Fig. 5 Pretreatment of rat plasma with rabbit antibody against rat IgM abrogates the innate antiviral activity in a dose-dependent manner. *a*, Rat plasma was pre-incubated with increasing doses of rabbit IgG raised against rat IgM (●) or of rabbit preimmune IgG (○) before being incubated with hrR3. Virus survival was measured in a lacZ-plaque neutralization assay. Horizontal axis, log scale. *b*, Plasma (unheated and diluted 1:8) from rats treated (+CPA) or not treated (–CPA) with CPA was pre-incubated with 60 mg/dl of rabbit antibody against rat IgM (+Anti-IgM) or vehicle before being incubated with hrR3.

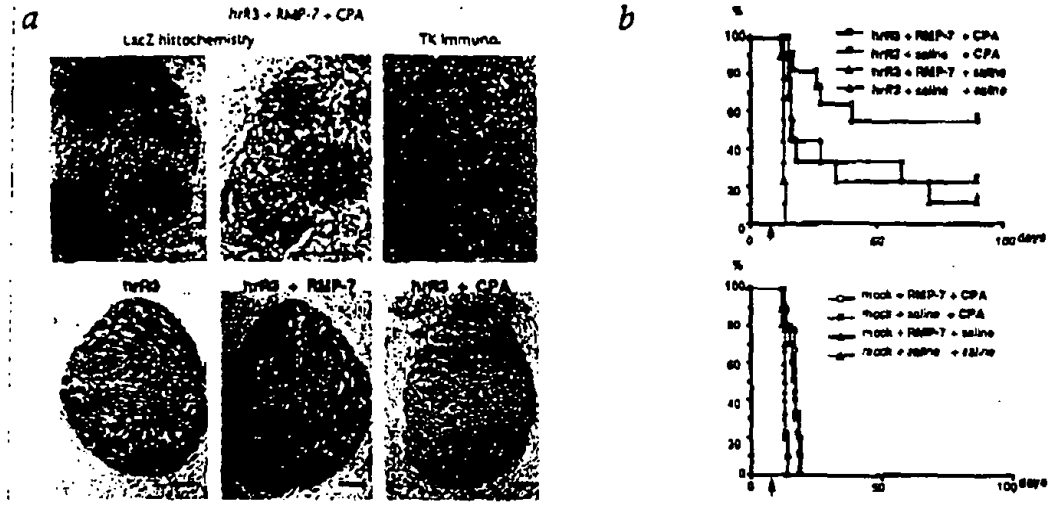


Fig. 6 Intravascular administration of oncolytic virus to a single human U87dEGFR glioma xenograft in rat brain. *a*, LacZ gene transduction and 'plaque' formation in the tumor 2 d after treatment with hrR3, RMP-7 and CPA (upper row) or hrR3 alone, hrR3 and RMP-7, or hrR3 and CPA (lower row). Upper right, a section adjacent to the sections in the other two upper panels was immunohistochemically processed using polyclonal antiserum against HSV thymidine kinase (TK). Scale bars represent 400 μm. *b*, Kaplan-Meier survival curves. Arrows, time of catheterization. The difference in survival between the group treated with CPA, hrR3 and RMP7 and the group treated with hrR3 and RMP7 was statistically significant: $P < 0.005$; the differences in survival for all groups was also significant: $P < 0.001$; Wilcoxon signed rank test.

manner; this innate activity affected complement function, but was not inactivated by the usual treatments used to remove complement; and depletion of calcium suppressed it. Preimmune IgM may contribute to this innate activity, by binding to virus and activating complement through the 'classical pathway'. Additional support for the involvement of complement is provided by the finding that its depletion with cobra venom factor also suppressed the innate antiviral action (Fig. 4*b*). Other possibilities, such as cell-mediated immune responses and their products, do not seem as likely, because they are elicited late after viral administration[17,28] and because we used athymic rats. The CPA-labile activity had to interact first with virus to protect cells from viral infection, thus indicating that it was not cytokine-based. Additional possibilities that remain the focus of investigation do not seem as likely, because they would require a previously unknown mechanism for CPA-mediated suppression of these molecules. These include a mannose-binding protein that binds foreign antigens and activates complement in a calcium-dependent manner ('lectin pathway'; ref. 21), and/or serum lipoproteins that are known to bind and inactivate HSV (ref. 29). Further evidence against involvement of serum lipoproteins in our experimental paradigm is their reported independence of calcium for HSV inactivation[29] and lack of known interactions with complement.

These findings are relevant to the use of oncolytic HSV vectors for the treatment of human tumors. Human plasma inactivated viral transducing ability to a degree similar to that of immunocompetent rat plasma. CPA partially reversed this activity, indicating that its administration would improve the efficacy of oncolytic viral therapy for cancer now in trials[14]. The presence of the innate activity in both human and rat plasma also indicated that it was unlikely to represent a natural antibody similar to the natural antibody against Gal found in human serum against murine retroviruses[30,31].

The current evidence favors a model in which intravascular (or intratumoral) injection of oncolytic virus results in viral aggregation by preimmune IgM. Although by itself the strength of this binding is relatively low (as it is present only in plasma at a dilution of 1:2; Fig. 2*a* and *b*), these aggregates are likely to activate complement through the calcium-dependent 'classical pathway', substantially enhancing the ability of plasma to inactivate virus, even at dilutions as high as 1:32 (Fig. 4). Moreover, large viral aggregates are likely to be inhibited in their ability to infect the cells and/or cross the tight junctions that compose the blood–brain barrier. CPA treatment of animals does not affect complement levels itself, but inhibits production of preimmune immunoglobulins

Table 2 LacZ cDNA expression and viral plaque formation in multiple intracerebral tumors 2 d (rats 1–8) and 4 d (rats 9–13) after intravascular administration of oncolytic virus.

Day after Treatment	Rat Number	% Intraneoplastic *LacZ* cDNA expression[a]		
		Tumor 1[b]	Tumor 2	Tumor 3
2	1	10.3 (4.4)[c]	2.6 (1.5)	0.6 (0.3)
	2	0	0	0
	3	0	-[d]	0
	4	4 (0.5)	0	0
	5	0	-[d]	1 (0.3)
	6	4 (0.9)	0	0
	7	1.5 (0.7)	0	0
	8	0	3.9 (1.9)	0
4	9	97.7 (1.9)	92.5 (2.5)	92.2(3.4)
	10	0	0	0
	11	89.3 (1.1)	82.2 (4)	82.1 (7.9)
	12	14.9 (2.3)	0	0
	13	0	65.1 (20.2)	0

CPA and RMP7 were administered to all rats. [a]Percentages were calculated by computer-assisted analysis of *lacZ*-expressing tumor area divided by total tumor area. [b]Tumor 1, 2, and 3: right frontal, right thalamic and left frontal cerebral neoplasms, respectively. [c]Values represent the mean, with s.e.m. in parentheses. [d]Tumor did not establish in this location in this rat.

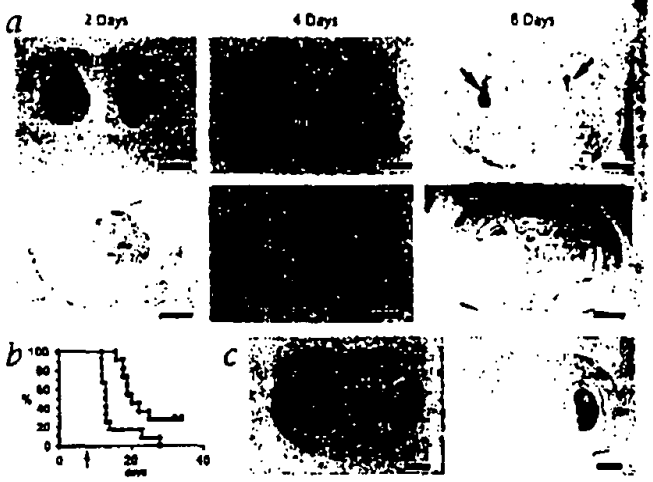


Fig. 7 Intravascular administration of oncolytic virus to human glioma xenografts established in brains of nude rats. *a*, LacZ gene transduction within three separate human glioma xenografts. Two sections from rats with three intracerebral tumors in both frontal lobes (upper row) and in the thalamus (lower row), 2, 4 and 8 d after administration of CPA, hrR3 and RMP-7. Scale bars represent 2 mm. Arrows, involuted tumors. *b*, Kaplan-Meier survival curves. Arrow, time of catheterization. The difference in survival between the groups treated with CPA, hrR3 and RMP7 (□) or hrR3 and RMP7 alone (■) was statistically significant: $P < 0.002$, Wilcoxon signed rank test). Rats in control groups (treated with mock injection and/or CPA) perished within 14 d of tumor implantation. *c*, Postmortem brain from one rat that failed treatment with CPA, hrR3 and RMP7, with a large intratumoral 'plaque' in an involuting tumor. Arrow, right frontal tumor disappeared. However, the left frontal tumor apparently was not infected and achieved a large volume, thus leading to the fatal outcome. Scale bars represent 2 mm.

(IgM and IgG) by B cells. CPA-mediated depression of circulating levels of IgM leads to decreased antiviral complement activity, thus increasing the survival of virus in tumors. In fact, in our experiments, survival of virus in CPA-treated rat tumors was higher than that in control tumors by 1×10 at 2 days and by 1×10^1 at 4 days, when the titer of neutralizing antibodies is expected to rise. At this point, CPA's initial 'permission' for virus survival in infected tumors, combined with its abrogation of neutralizing antibody formation, sets up the intraneoplastic propagation of oncolytic virus' progeny and the involution of tumors seen at the later stages of our experiment (days 4–8). The effect of complement inhibitors (such as cobra venom factor) as well as the effect of combinations of cobra venom factor with CPA on brain tumor infection by the oncolytic virus need to be determined.

Herpes simplex viruses have evolved several mechanisms to escape recognition by the immune system, such as the inactivation of the C3 component of complement by glycoprotein C of HSV and the inactivation of some humoral antiviral functions by binding of non-immune and immune Fc IgG by glycoproteins E and I (refs. 32,33). Not much is known about the ability of HSV to inactivate IgM. The findings here may be generalized to other HSV strains, because a similar innate activity inactivated a HSV mutant from strain F and a HSV amplicon derived from strain 17, but not a VSV-G pseudotyped retroviral vector (K.I. and E.A.C., unpublished results).

Oncolytic viruses can provide selective anticancer effects in experimental tumor models[2,4–11]. The addition of anticancer genes to the viral genome can also augment oncolysis[7–9]. In fact, an ICP6 mutant that expresses the CYP2B1 gene responsible for the activation of the 'pro-drug' cyclophosphamide into its active anticancer and immunosuppressive metabolites has been generated[3]. This mutant and other HSV mutants should be safer and more effective than hrR3 for further testing of intravascular administration in appropriate primate models. Ultimately, 'superselective' arterial catheterization techniques provide an avenue for safe and efficacious clinical trials in patients afflicted with refractory, multifocal tumors in an organ.

Methods

HSV-1 vector stocks. The virus used here was the genetically engineered HSV mutant hrR3, derived from HSV-1 KOS, which has a disruption of the UL39 gene through insertion of the *Escherichia coli* lacZ gene under the control of the ICP6 promoter[14–16]. The oncolytic selectivity of the virus has been described[2,4–6,11]. Viral stocks were generated in African green monkey kidney cell culture (Vero) and titered by plaque assays as described[17]. As a control, a 'mock-infection' extract was prepared from vehicle-infected cells using the same procedures. A total of 1×10^8 PFU in 100 µl were introduced into the carotid artery as a bolus injection. The virus was also grown in 9L cells and U87dEGFR cells and passaged three times before antiviral activity assay.

Cell culture. U87dEGFR cells were a gift from H.-J.Su Huang (University of California at San Diego). This cell line was established by retroviral transfer of a mutant epidermal growth factor receptor (de 2-7 EGFR) into U87 human glioblastoma cell line, enhancing its tumorigenic capacity in the brains of nude mice[18]. Human U87dEGFR cells and rat 9L gliosarcoma cells were propagated at 37 °C in an atmosphere containing 5% CO2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml G418 (for the U87dEGFR cells).

Assays for antiviral activity in plasma. In addition to collecting plasma from rats, we collected human plasma from 20 patients before and 2 days after they underwent chemotherapy with CPA. Plasma samples were incubated for 30 min at 56 °C and then serially diluted with PBS (starting with a 1:2 dilution). Plasma treated with EGTA-Mg contained 10 mM EGTA and 7 mM $MgCl_2$. Plasma (100 µl) was incubated with 2×10^6 PFU of hrR3 for 1.5 h at 37 °C and applied onto 4×10^4 Vero cells in 24-well plates. Then, 16 h later, cells were fixed and stained for β-galactosidase activity. Neutralizing antibody titers were determined by the highest dilution of heated plasma that reduced the number of β-galactosidase-positive cells by 50% or more, compared with control. Antiviral activity of plasma is shown as percentage of the number of β-galactosidase-positive cells, compared with control. In pilot experiments, the ability of hrR3 to form a lacZ-positive plaque in Vero cells (4–5 days) was essentially identical to its ability to initially transduce a cell. For antibody neutralization studies, rabbit IgG raised against rat IgM and rabbit preimmune IgG were purchased from Accurate Chemical & Scientific (Westbury, New York). Increasing doses of antibodies were then incubated with unheated plasma from nude rats, diluted 1: 4 (final plasma dilution 1:8) for 1 h at room temperature before incubation with virus for 1.5 h at 37 °C. The mixture was then used to infect Vero cells to assay for effects on viral activity.

Animal studies. Adult male Fischer 344 rats or female nude rats (mu/mu) were anesthetized with an intraperitoneal injection of 0.5 ml of 0.9% NaCl containing 12.5 mg of ketamine and 2.5 mg of xylazine. After rats were immobilized in a stereotactic apparatus, a linear skin incision was made over the bregma, and a 1-mm burr hole was drilled in the skull approximately 1 mm anterior to and 2 mm lateral to the bregma on the right side. For the multifocal tumor model, two additional burr holes were drilled. The first was 1 mm anterior to and 2 mm lateral to the bregma on the left side, and the

other was 3 mm posterior to and 2 mm lateral to the bregma on the right side. 9L cells (4 × 10⁴) or U87dEGFR cells (2 × 10⁵), in a 2-μl volume, were injected at a depth of 3.5 mm from the dura, using a 5-μl Hamilton syringe.

Then 12 d later for 9L or 8 d later for U87dEGFR, 100 mg/kg cyclophosphamide or vehicle was injected intraperitoneally, followed by intra-arterial catheterization. The catheterization technique used was essentially identical to that described[11]. RMP-7 (1.5 μg/kg) or vehicle was infused for 10 min. Midway through the infusion, a 100-μl bolus of virus (1 × 10⁹ PFU) or vehicle was given. For virus distribution studies, virus (2 × 10⁷ PFU in 2 μl) was inoculated stereotactically into 8-day-old intracerebral U87dEGFR tumors and then rats were killed 1, 2, 3 or 4 d later. Their brain tumors were aseptically explanted, weighed and then minced. Tissue was homogenized in HBSS containing 1 mg/ml collagenase/dispase (Boehringer) and then incubated at 37 °C for 1 h. Virus was collected from the tissue by freezing and thawing it repeatedly and using ultrasonication, and then virus was assayed by plaque assays on Vero cells. For lacZ-expression and toxicity assays, rats were anesthesized and then perfused with an intracardiac infusion of a solution containing 4 % neutral paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brain, liver, lung, kidney and heart were removed and placed in 4% PFA–PBS overnight at 4 °C. They were then transferred to 30% sucrose in PBS for 2 d, frozen with liquid nitrogen and stored at –80 °C. For survival studies, rats were observed twice daily and if they showed neurologic impairment (inability to feed, drink or move), at which time they were killed. For antiviral activity assays, blood samples were drawn from the heart and stored at –80 °C. Blood samples were also obtained from rats that had been injected intraperitoneally with CPA on day -2 and/or with cobra venom factor (Quidel, San Diego, California) on day –1 at a dose of 60 U/kg and on day 0 at 20 U/kg (days numbered with respect to the day rats were killed). Statistical analyses used the statistical software program StatXact and/or Sigmaplot using Kaplan-Meier survival estimation and the Wilcoxon signed rank test for significance.

Histochemistry for virus distribution assay. Brains were sectioned on a cryostat and sections 20 μm in thickness were air-dried at room temperature. Sections were stained either by histochemistry using the X-Gal substrate, as described[12], or by immunohistochemistry using rabbit polyclonal antibodies against highly purified HSV-TK (a gift from W. Summers, Yale University School of Medicine) and were counter-stained with either neutral red or hematoxylin.

Quantitative analysis of virus distribution by a computer-assisted method. Three sections of brain adjacent to the injection site randomly selected from each rat were analyzed using an Olympus BX60 microscope. The sections were scanned by Sony 3-chip Color Video Camera at ×20 magnification, and the entire tumor area and β-galactosidase-positive area were measured using Image Pro Plus Imaging Software.

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